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PHENOTYPIC AND FUNCTIONAL CHARACTERISATION OF MYELOID AND PLASMACYTOID DENDRITIC CELLS

A thesis submitted in part fulfilment of the requirement for the degree of

Doctor of Philosophy

By

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Abstract

Cord blood transplantation (CBT) is an alternative to bone marrow transplantation (BMT) and is associated with a reduced severity of graft versus host disease (GvHD). Recipient and donor dendritic cells (DCs) are involved in the development of GvHD, and two main subsets, myeloid DCs (MDCs) and plasmacytoid DCs (PDCs), with different immunological functions, have been identified in adult peripheral blood (APB). In this thesis, the presence, phenotype and functional characteristics of these two DC subsets were investigated in CB.

Absolute cell counts, the expression of a number of markers and the endocytic capacities were studied on highly purified MDCs and PDCs by flow cytometry. The cytokine profile of stimulated DC subsets was determined using a multiple cytokine detection system. The allostimulatory capacity was studied *in vitro* and the cytokine production of the cultures was also assessed.

The results showed the presence of MDCs and PDCs in CB, with PDCs as the predominant population. Surface marker expression differed between CB DC subsets, and levels of expression were generally lower compared to APB. Stimulated CB MDCs and PDCs secreted Th1 and Th2 cytokines, respectively. CB MDCs had a higher endocytic capacity than PDCs, as shown for APB. The allostimulatory capacity of CB MDCs was higher than CB and APB PDCs, but lower than APB MDCs. Comparisons of fresh and frozen cells revealed a reduced allostimulatory capacity of frozen CB DC subsets, not apparent in APB. There was preferential secretion of Th2 cytokines following allogeneic stimulation with CB MDCs and PDCs.

The results showed different phenotypic and functional characteristics of CB MDCs compared to PDCs. The studies confirmed the immature status of CB DCs and their capacity to mount adult level responses when appropriately stimulated. Therefore, these *in vitro* studies may help to explain the reduced incidence and severity of GvHD following CBT.

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Abbreviations

APB	=	Adult peripheral blood
*a*GvHD	=	Acute graft versus host disease
ALL	=	Acute lymphoblastic leukaemia
APC	=	Antigen presenting cell/Allophycocyanin
AutoMACS	=	Automated magnetic cell sorting system
β2M	=	Beta-2-microglobulin
BCR	=	B cell receptor
BDCA	=	Blood dendritic cell antigen
BM	=	Bone marrow
BMT	=	Bone marrow transplantation
BSA	=	Bovine serum albumin
CB	=	Umbilical cord blood
CBT	=	Umbilical cord blood transplantation
CFSE	=	5- and 6-carboxyl fluorescein disuccinyl ester
CLIP	=	Class II associated invariant chain peptide
CD	=	Cluster of differentiation
*c*GvHD	=	Chronic graft versus host disease
CML	=	Chronic myeloid leukaemia
CMV	=	Cytomegalovirus
CpG ODN	=	Cytidine-guanosine oligodinucleotides
cpm	=	Counts per minute
CTL	=	Cytotoxic T lymphocyte
C-type	=	Calcium-dependent
DC	=	Dendritic cell
DMSO	=	Dimethylsulphoxide
DNA	=	Deoxyribonucleic acid
DNTPs	=	Deoxynucleotide triphosphates
dsRNA	=	Double stranded ribonucleic acid
EBV	=	Epstein barr virus
EDTA	=	Ethylenediaminetetraacetic acid
ELISA	=	Enzyme linked immunosorbent assay

ER	=	Endoplasmic reticulum
FACS	=	Fluorescence-activated cell sorting
FCS/FBS	=	Fetal calf/bovine serum
FITC	=	Fluorescein isothiocyanate
Flt3L	=	Fms-like tyrosine kinase 3 ligand
G-CSF	=	Granulocyte-colony stimulating factor
GM-CSF	=	Granulocyte-macrophage colony stimulating factor
GvHD	=	Graft versus host disease
GvL	=	Graft versus leukaemia
^{3}H Thymidine	=	Methyl-^{3}H-Thymidine
HIV	=	Human immunodeficiency virus
HLA	=	Human leukocyte antigen
hr	=	Hours
HSC	=	Haematopoietic stem cell
HSCT	=	Haematopoietic stem cell transplantation
ICAM	=	Intercellular adhesion molecule
IFN	=	Interferon
IL	=	Interleukin
Ii chain	=	Invariant chain
KCl	=	Potassium chloride
L	=	Ligand
LC	=	Langerhans cell
Lin	=	Lineage
LFA	=	Leucocyte function-associated antigen
LN	=	Lymph node
LPS	=	Lipopolysaccharide
MAPK	=	Mitogen activated protein kinase
MACS	=	Magnetic activated cell sorting system
MDC	=	Myeloid dendritic cell
MDDC	=	Monocyte derived dendritic cell
MHC	=	Major histocompatibility complex
MLR	=	Mixed Lympocyte Reaction
MNC	=	Mononuclear cell
MoAbs	=	Monoclonal antibodies

MR	=	Macrophage mannose receptor
MW	=	Molecular Weight
NF-κB	=	Nuclear Factor-KappaB
NK	=	Natural killer cell
NP-1	=	Neuropilin-1
PAMP	=	Pathogen-associated molecular pattern
PBMNC	=	Peripheral blood mononuclear cell
PBS	=	Phosphate buffered saline
PBSC	=	Peripheral blood stem cell
PBSCT	=	Peripheral blood stem cell transplant
PCR	=	Polymerase chain reaction
PDC	=	Plasmacytoid dendritic cell
PDN	=	Peptidoglycan
PerCP	=	Peridinin-chlorophyll-protein
PE	=	Phycoerythrin
PFA	=	Paraformaldehyde
PHA	=	Phytohaemagglutinin
PMA	=	Phorbol 12-myristate 13-acetate
Pre-Tα	=	Pre-T-cell antigen receptor α chain
PRR	=	Pattern recognition receptor
RPMI	=	Roswell Park Memorial Institute
RT	=	Room temperature
RT-PCR	=	Reverse transcription-polymerase chain reaction
SDS	=	Sodium Dodecyl Sulphate
SSP	=	Sequence specific primers
TAP	=	Transporters associated with antigen processing
TCR	=	T cell receptor
Th1/2	=	T helper 1/2
TI	=	Thymus-independent
TLR	=	Toll-like receptor
TNF	=	Tumour necrosis factor
T_{reg}	=	Regulatory T cell
VEGF	=	Vascular endothelial growth factor

Chapter I

INTRODUCTION

1.1 The immune response

The immune system consists of a complex cellular network with the primary function of protecting against pathogens by initiating an immune response without causing damage to self. The main properties for an effective immune response include (a) the ability to recognise antigens with a high degree of specificity, (b) the development of memory, (c) the induction and maintenance of tolerance against self-destructive immune responses and, (d) the ability to launch distinct immune responses to different types of pathogens (Pulendran, 2005).

The immune response can be divided into two phases, innate immunity and the adaptive immune response:

Innate immunity provides the first line of defense against a pathogen and is the basic resistance to a disease. It is comprised of anatomic (skin), physiologic (temperature), endocytic and inflammatory defensive barriers. An invading pathogen can be recognised via special receptors referred to as pattern recognition receptors (PRRs), expressed by antigen presenting cells (APCs) (Banchereau et al., 2000). These receptors bind pathogen associated molecular patterns (PAMPs) on microbes thus allowing the immune system to distinguish between self and non-self (Janeway, Jr. and Medzhitov, 2002; Medzhitov and Janeway, Jr., 2002) and triggering 'danger signals' (Matzinger, 1994; Kaisho and Akira, 2001). Examples of PRRs include the Toll-like receptors (TLRs), originally described for their homology with the *Drosophila* toll receptor (Medzhitov et al., 1997), the macrophage mannose receptor (MR) and scavenger receptor, both of which are C-type lectins (Iwasaki and Medzhitov, 2004).

Adaptive immunity provides immunity following initial infection. An effective adaptive immune response requires interplay between two major groups of cells, the lymphocytes and the APCs. The main effectors of adaptive immunity are the lymphocytes. Lymphocytes recognise and interact with antigens through membrane receptors specific for foreign pathogens. The two populations of lymphocytes include B cells and T cells. B cells mature in the bone marrow (BM) and express membrane-bound antibody molecules important for humoral

immunity. The antibody and antigen react with specificity and the cell rapidly divides differentiating into a plasma cell which then produces and secretes antibody. In contrast, T cells mature in the thymus, where they rearrange and express the T cell receptor (TCR) for cell mediated immunity. The antibodies on B cells recognise intact antigen. However, the TCRs on naïve T cells require pathogen derived peptides to be associated with Major Histocompatibity Complex (MHC) molecules in order for the T cells to be activated, to proliferate and differentiate into memory and effector T cells. Another difference between the two lymphocytes is that all B cells have the same general function of making immunoglobulins (Ig), whereas the different types of T cells (e.g. CD4 and CD8) have distinctive functions (e.g. help and cytotoxicity).

1.2 B cells

B cell development initially occurs in the fetal liver (week 8 or 9 of gestation) and subsequently in the BM, where B cell production continues throughout life. During the early stages of B cell development, there is gene rearrangement to produce a functional Ig molecule, which then serves as the B cell receptor (BCR) on the cell surface of B cells. A heavy (H) chain gene is first assembled during B cell differentiation. It is transcribed and the translated protein can pair with a light (L) chain that is also a product of recombination. A B cell initially expresses IgM as its BCR and it is the first antibody produced prior to an immune response. Later in the immune response, IgG, -A, or -E is expressed and this change is referred to as isotype switching, involving DNA rearrangement. Class switching allows the body to produce antibodies with different effector functions.

The BCR binds antigen and delivers it to intracellular sites where it is degraded and presented to the surface as peptide bound to HLA class II (Roosnek and Lanzavecchia, 1991). The peptide-HLA class II complex is recognised by CD4 T helper (Th)2 cells delivering activating signals to B cells, initiating B cell proliferation. When an activated B cell ceases to proliferate, it can differentiate into an antibody secreting plasma cell and later into a memory B cell. Some microbial thymus-independent (TI) antigens, e.g. lipopolysaccharides (TI-1) or

bacterial polysaccharides (TI-2) can activate B cells in the absence of T cell help, through binding of part of the antigen to a receptor, e.g. TLR or crosslinking of the membrane antibody by a polymeric antigen, providing a rapid response to pathogens. TI antigens induce limited isotype switching and do not induce memory B cells but responses to these antigens have a crucial role in host defence against pathogens whose surface antigens cannot elicit peptide-specific T cell responses. However, T cell help is required for Ig class switching resulting in more variability and versatility. The additional signals from Th cells, but also macrophages and DCs, are necessary for B cell growth and differentiation.

B cells are divided into two subsets, B1 and B2 cells (Miller and Phillips, 1975). B1 cells comprise 5% of all B cells, appearing during fetal development and provide an early response to antigens. B1 cells express CD5 and are present in the peritoneal and pleural cavities in adults where they express IgM and IgD on their surface responsible for T cell independent antibody production. The B2 type B cell is the major population located mainly in the secondary lymphoid organs and in the circulation. The conventional B2 cells express CD19 and CD40, which are important in signalling. CD40 is a member of the TNF receptor (TNFR) family of cytokine receptors important in APC function (Banchereau et al., 1995; Gruss et al., 1996). Binding of CD40 to its ligand (CD40L), an important T cell effector membrane molecule, helps drive the resting B cells into the cell cycle and is essential for responses to thymus-dependent antigens (Noelle et al., 1992). CD40 increases the expression of costimulatory molecules (Goldstein and Watts, 1996) on B cells which provide signals that sustain T cell growth and differentiation, enhancing T and B cell interaction (Swain, 1994). IL-4 produced by Th2 cells, and CD40L synergise in driving clonal expansion that precedes antibody production (Smith et al., 2000).

1.3 The T cell repertoire

T cell maturation within the thymus also involves a series of random rearrangements of gene segments encoding the TCR, which generates enormous diversity. During maturation, thymocytes differentiate along one of two

developmental pathways (Palacios et al., 1989). A minority of thymocytes rearrange the γδ chain genes and develop into $CD3^+$ γδ T cells. The majority of double negative cells ($CD4^-CD8^-$) rearrange the TCR α and β chain genes and express both CD4 and CD8. The double positive cells develop into $CD4^+CD8^-$ or $CD8^+CD4^-$ T cells (Guidos et al., 1990; Hugo et al., 1990). The two subpopulations of T cells, Th cells and cytotoxic T lymphocytes (CTLs) are, therefore, distinguished by the presence of CD4 or CD8 membrane glycoproteins, respectively. Th cells may function as Th1 lymphocytes, which secrete cytokines that support the cellular immune response, or as Th2 cells favouring humoral immunity. CTLs recognise and kill virally infected cells through apoptosis by recognising foreign peptides that are bound to HLA class I molecules. A further subset, the $CD4^+CD25^+$ regulatory T cell (T_{reg}) is important in maintaining peripheral tolerance to self-antigens, preventing self-reactive T cell activation through the action of suppressing the proliferation of $CD4^+$ and $CD8^+$ T cells (Sakaguchi et al., 1995; Sakaguchi, 2000; Karim et al., 2002). T_{regs} can inhibit development of GvHD, prevent anti-tumour responses and regulate feto-maternal tolerance (Sutmuller et al., 2001; Cohen et al., 2002; Hoffmann et al., 2002; Taylor et al., 2002; Phan et al., 2003; Aluvihare et al., 2004).

The different classes of T cells are activated depending on the foreign peptides presented by the MHC molecules on the APCs to the TCR. The $CD8^+$ T cells have specificity for MHC class I-peptide complexes and therefore, are Class I MHC-restricted. The T cell subset expressing CD4 recognises antigens associated with Class II, and is Class II MHC-restricted. This phenomenon of MHC restriction (Zinkernagel and Doherty, 1997) was introduced through thymic selection, a process ensuring that only T cells with receptors capable of recognising antigens associated with self-MHC molecules would be able to mature (Kisielow and von Boehmer, 1991; Starr et al., 2003). Cells with low or no affinity for self-MHC fail to receive positive signals needed for survival and are ignored. Cells with 'high' affinity for self-peptide are perceived as dangerous and are deleted through the process of negative selection. Thymic dendritic cells (DCs) are one of the most effective triggers of negative selection (Zinkernagel and Althage, 1999; Brocker et al., 1997; Brocker, 1999). The cells with a 'moderate' affinity for self-MHC-

peptide complexes are positively selected for via specialised thymic cortical epithelial cells to populate the immune system.

Due to the way in which T cells are selected, where there is TCR bias for self-MHC recognition, any foreign cell would be rejected due to T cell alloreactivity. The high frequency of alloreactive T cells is explained by the fact that a TCR is specific for a foreign antigen associated with self-MHC molecules. Although, the TCR can also crossreact with allogeneic MHC molecules, i.e. the same TCR can recognise both the allogeneic MHC-peptide complex and the foreign peptide, plus self-MHC molecule, which has implications in the transplantation setting. The idea of the recognition of self and non-self was supplemented by a newer model proposing that the innate immune system recognises danger delivering signals to T cells enabling them to develop immunity or tolerance (Matzinger, 1994; Medzhitov and Janeway, Jr., 2000).

1.4 The MHC

The MHC is a collection of genes arranged within the short arm of chromosome six in humans and is referred to as the human leucocyte antigen (HLA) complex. This complex is important in determining whether transplanted tissue will be accepted as self or rejected as foreign. HLA genes are highly polymorphic and inherited codominantly in strong linkage. As HLA molecules are important in the induction of the immune response, the polymorphism is most likely due to selection for increased protection against pathogens (Apanius et al., 1997).

HLA molecules are antigen processing and presenting molecules, binding to a broad spectrum of antigens which are degraded and presented as antigenic peptides to T cells. Antigenic peptides are generated through two distinct pathways, the 'HLA class I antigen processing pathway' which involves endogenous antigens such as viruses and bacteria, and the 'HLA class II antigen processing pathway' where exogenous antigen produced outside the host enters the cell by endocytosis or phagocytosis, is processed and then presented at the cell surface (Janeway et al., 2005).

The HLA loci encode two classes of membrane molecules, HLA class I and class II. The classical HLA class I molecules are encoded by A, B and C regions and the non-classical by E, F and G. Most nucleated cells express HLA class I molecules. HLA class I molecules consist of three extracellular α heavy chain domains (α1, α2 and α3) noncovalently linked to β2-microglobulin (β2M) with the α3 domain (Figure 1.1). The α3 domain is anchored in the cell membrane by its hydrophobic transmembrane segment and hydrophilic cytoplasmic tail. The α1 and α2 domains are the most polymorphic, and they form the peptide-binding groove, where short peptides (8-10 amino acids) bind. The main difference with the non-classical molecules is the limited polymorphism and restricted tissue distribution. For example, the nonclassical HLA-G molecule is expressed at the feto-maternal interface on extravillous cytotrophoblasts (Kovats et al., 1990), fetal endothelial cells (Blaschitz et al., 1997) amniotic fluid (Hammer et al., 1997) and also on certain tumour cells (Paul et al., 1999). HLA-G is the product of a class Ib gene and is thought to display a capacity of presenting antigenic peptides (Lee et al., 1995). Alternative splicing of the HLA-G primary transcript generates four membrane-bound isoforms (HLA-G1 to -G4) and three soluble isoforms (HLA-G5 to -G7) (Figure 1.2) (Paul et al., 2000). Membrane bound HLA-G1 has been shown to suppress $CD4^+$ T cell proliferation (Bainbridge et al., 2000a), and soluble HLA-G1 (i.e. HLA-G5) can induce the apoptosis of $CD8^+$ T cells (Le Bouteiller and Solier, 2001). More recently, HLA-G molecules have been shown to be involved in the inhibition of both T and natural killer (NK) cell-mediated cytolysis through their interaction with inhibitory receptors such as Ig-like transcript (ILT)2 and ILT4 (Rajagopalan and Long, 1999; Riteau et al., 2001; Contini et al., 2003).

There are three main kinds of classical HLA class II molecules, HLA-DR, HLA-DP and HLA-DQ. Each has two genes which encode the α and β chains of the molecule. The HLA class II molecule consists of two external noncovalently associated domains (α1 and α2; β1 and β2) (Figure 1.1). The extracellular domains (α1 and β1) of the HLA class II molecule also form a peptide-binding

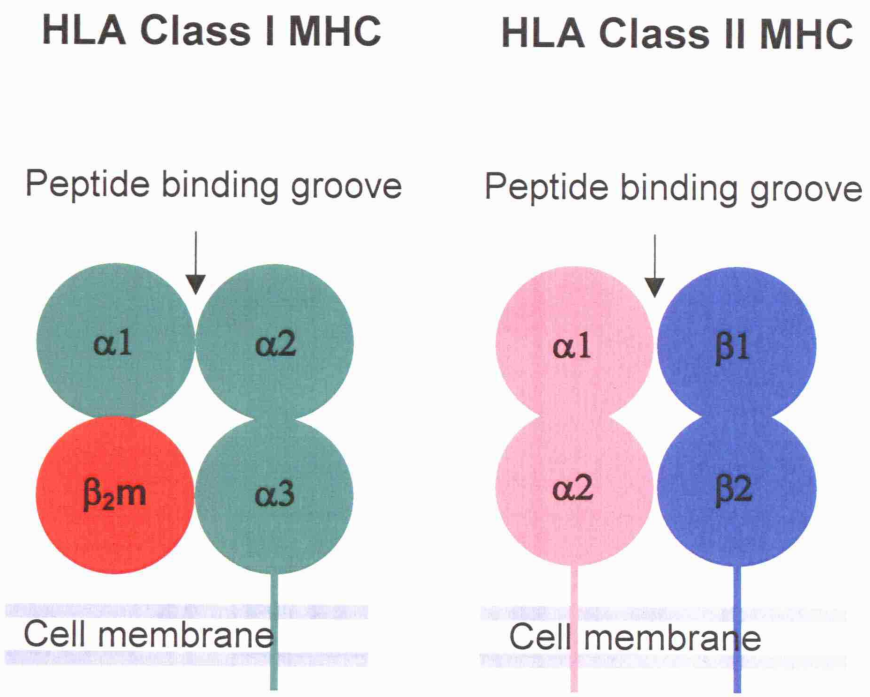


Figure 1.1. The structure of the HLA Class I and II molecules .

There are differences in the structure of the two HLA molecules, HLA class I and class II. HLA class I molecules consist of three α chain domains (α1, α2 and α3) and β2-microglobulin (β2m). HLA class II molecules are represented by a set of α1 and α2 chains. The peptide binding groove has also been highlighted.

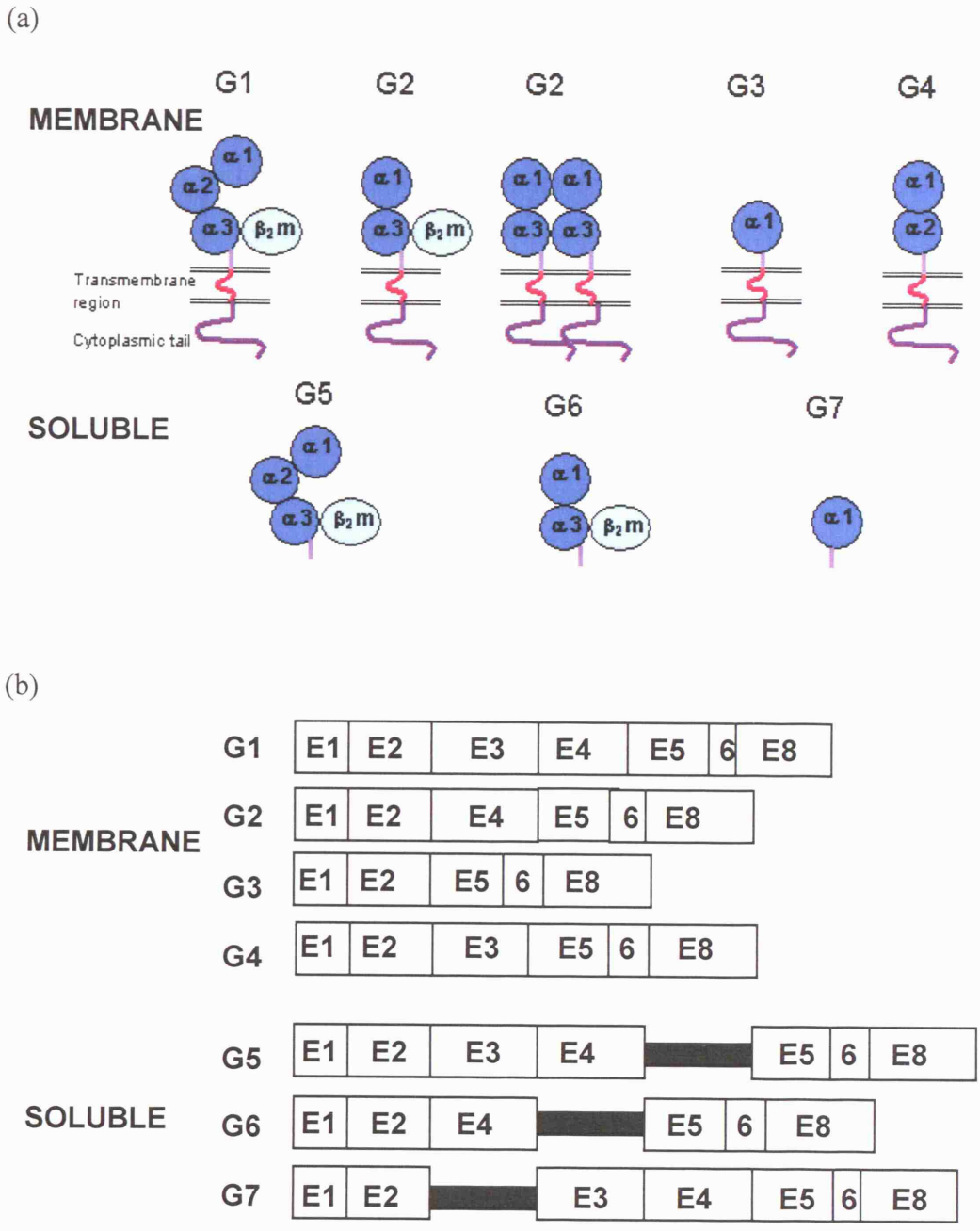


Figure 1.2. The structure of the nonclassical class I molecule, HLA-G.

(a) represents the protein structure of the different isoforms of HLA-G. HLA-G1 and G5 resemble the classical HLA class I molecules associating with β_2M. G2 and G6 lack the α2 domain. G3 and G7 consist of the α1 domain alone and G4 is made up of α1 and α2 domains; (b) refers to the mRNA splice variants encoding four membrane bound (HLA-G1 and G4) and three of the soluble (G5 to G7) isoforms of HLA-G (Bainbridge et al., 2001).

groove, but it is open at both sides enabling the binding of longer peptides (13-25 amino acids). An additional chain, the invariant (Ii) chain, is transiently associated with the class II heterodimer during transport to the cell membrane. The nonclassical HLA class II molecules include HLA-DM and HLA-DO. HLA-DM is present in all APCs, but HLA-DO is mainly expressed in B cells. HLA-DO has a modulatory function on the activity of HLA-DM (Alfonso and Karlsson, 2000). The function of HLA-DM is to load peptide onto HLA class II molecules by catalysing the release of CLIP. It also functions to stabilise empty HLA class II, which would otherwise aggregate and degrade in the absence of the appropriate peptides (Denzin et al., 1996; Kropshofer et al., 1997). HLA-DM does not bind peptides, but acts as a 'peptide editor', removing unstable peptides and favouring presentation of high-stability peptides, hence narrowing the spectrum of peptides to be processed and presented (see section 1.6.5) (Alfonso and Karlsson, 2000).

HLA class II molecules are glycoproteins expressed by specialised cells that function as APCs. APCs are the primary producers of HLA class II molecules under normal conditions. However, under the influence of other stimuli, i.e. cytokines or inflammation, other cells such as endothelial cells and activated T cells can also express HLA class II molecules.

When an infection is recognised by PRRs on APCs, the antigen is taken up by the cell, processed, and the antigenic peptide is presented on the cell surface to the TCR on T cells, which are subsequently activated. The signals provided by the peptide-HLA complex, adhesion and costimulatory molecules and cytokines from APCs induce T cell activation (Figure 1.3). The initial contact between the peptide-HLA complex on an APC and the TCR on a T cell constitutes signal 1 of the process of antigen presentation. When APCs initially interact with T cells, cell adhesion molecules are involved. Intercellular adhesion molecules (ICAMs) are important for adhesion between APCs and T cells. For example, the binding of T cells to DCs occurs through interactions between LFA-1 and CD40L on T cells and ICAM-1 and CD40 on APCs, respectively (Figure 1.3). Signals derived from costimulatory molecules are collectively referred to as signal 2. CD80 and CD86 glycoproteins are the best-characterised costimulatory molecules expressed on APCs that bind to the receptors, CD28 and CTLA4 on T cells, essential for the

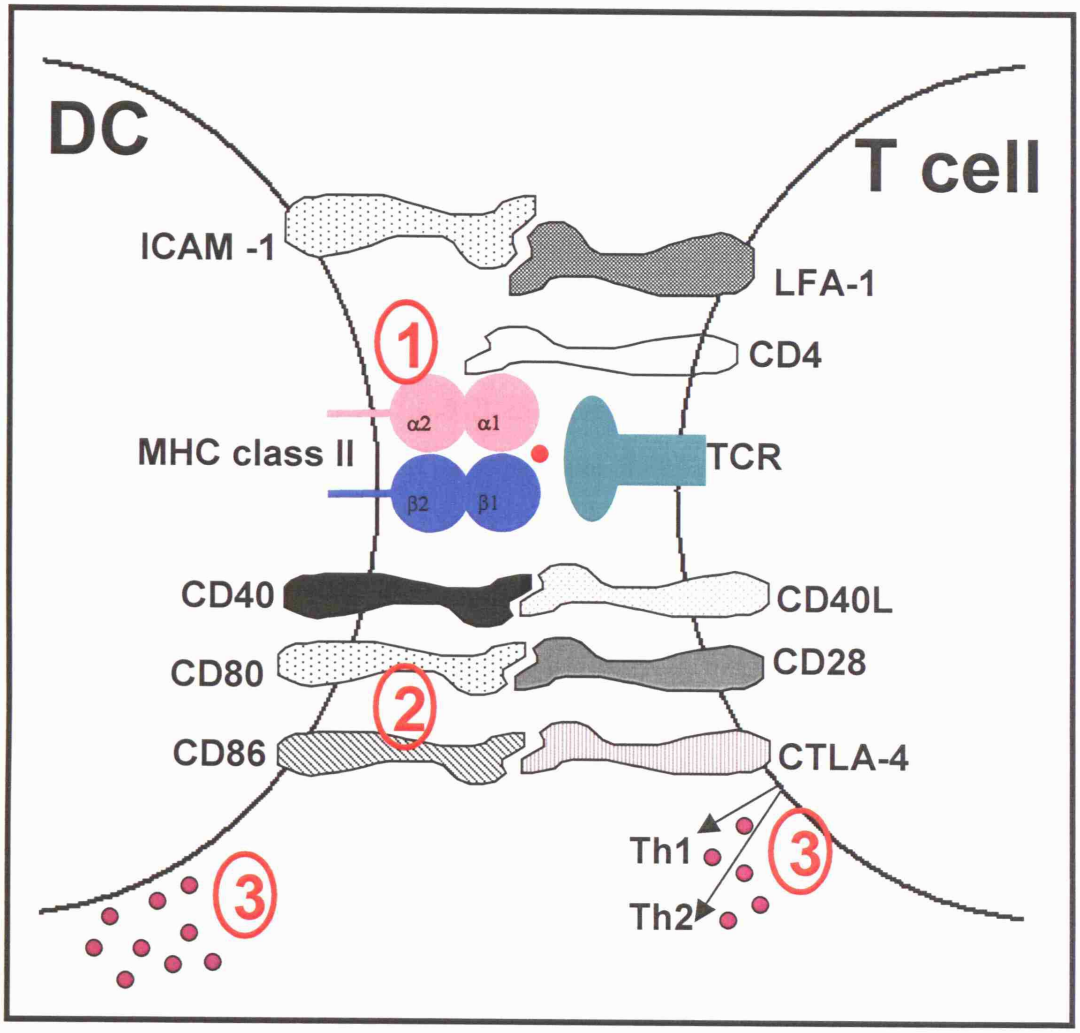


Figure 1.3. The interaction of a dendritic cell with a T cell following antigen encounter.

The various steps involved in antigen presentation include signal (1) where the MHC-peptide complex on APCs interacts with the TCR on T cells. Signal (2) follows, where adhesion and costimulatory molecules are upregulated by both APCs and T cells. Finally, signal (3) is represented by the secretion of cytokines by APCs and following T cell activation.

clonal expansion of the T cells (Lenschow et al., 1996; Grewal and Flavell, 1998; Chirathaworn et al., 2002; Kandula and Abraham, 2004). Therefore, costimulation is initiated by CD28 binding to CD80/86. Delivery of signal 1 without signal 2 may involve the mechanism of maintaining tolerance (Schwartz, 1990; Matzinger, 1994; Kundig et al., 1996). Activated APCs secrete cytokines and this constitutes signal 3. DCs secrete cytokines such as IL-12 and IFNα, which then instruct T cell polarisation (Th1 or Th2). An activated T cell becomes an effector cell and responds by also secreting cytokines, which activate other immune cells. Th1 and Th2 cells release different sets of cytokines, defining their distinct actions in the immune response. Th1 cells secrete IL-2 and IFNγ during inflammatory responses supporting the function of NK and $CD8^+$ T cells (Banchereau and Steinman, 1998; Tarte and Klein, 1999). IL-4, IL-5, IL-10 and IL-13 production characterises Th2 cells, which subsequently induce a classical antibody response via B cell involvement (Abbas et al., 1996).

1.5 Antigen presenting cells

A variety of cells can function as APCs and their distinguishing feature is the expression of HLA class II molecules. The various APCs that include DCs, macrophages and B cells, are all differentially distributed. They vary in their antigen presenting ability, being adapted to present distinct types of pathogens and pathogenic products (Janeway et al., 2005). DCs can efficiently process and present an array of antigens, whereas the other APCs are more specialised and specific in their function. B cells can efficiently internalise soluble molecules through their cell surface Ig receptors, whereas, the macrophage ingests foreign particulate matter by phagocytosis. Macrophages are equipped to scavenge and remove dead or dying cells, which are abundant in self antigens.

The surface expression of HLA and costimulatory molecules is higher in DCs than B cells or macrophages. DCs constitutively express both HLA and costimulatory molecules. B cells express HLA class II molecules, which are upregulated to facilitate the presentation of a high density of peptide-self HLA class II complexes at the cell surface enabling targeting by antigen-specific T cells. B cells can only

express costimulatory molecules once activated. The lack of costimulation leads to anergy or unresponsiveness, where self-reactive T and B cells are eliminated or inactivated during development. Macrophages express very few HLA class II molecules and expression of CD80 and CD86 is only evident after ingestion of micro-organisms and recognition of PAMPs.

DCs have a unique function of stimulating naïve T cells, a role which neither B cells or macrophages can perform (Young, 1990). Due to this unique ability to stimulate resting naïve T cells, DCs have been referred to as the most potent APCs (Hart, 1997). In addition, DCs can present antigen via both the HLA class I and class II pathway. The generation of a T cell response requires recognition and uptake of antigen by DCs with the capacity to process antigen for presentation to naïve T cells. DCs therefore, possess a function of initiating and regulating the immune response (Hart, 1997). This is a major reason for studying DCs rather than any other APC.

1.6 Dendritic cells

DCs were initially visualised as Langerhans cells (LCs) in the skin 1868 and then described in the early 1970s as a population of dendritic-shaped cells present in the spleen (Steinman and Cohn, 1973). DCs are BM-derived cells widely distributed *in vivo*, present as immature cells in the skin, mucosal surfaces, in primary and secondary lymphoid organs and most nonlymphoid tissues as well as circulating in the periphery (Cella et al., 1997; Banchereau and Steinman, 1998; Reis e Sousa et al., 1999; Moser and Murphy, 2000). DCs originate from both myeloid and lymphoid progenitor cells in the BM, circulate in the blood as precursors prior to migrating into peripheral tissues where they give rise to LCs, dermal and interstitial DCs. Blood DCs represent approximately 0.5-1% of the total leucocyte population in peripheral blood mononuclear cells (PBMNCs) (Fearnley et al., 1999).

DCs are specialised cells that act as sensors and messengers, sampling the environment of peripheral tissues in an immature state (Banchereau and Steinman,

1998). DCs can assimilate various signals and convey them to lymphocytes which leads to the eradication of invading microbes. Therefore, DCs have a dual role. Firstly, upon encountering microbes or tissue damage, immature DCs recognise pathogens through PRRs and actively endocytose them using receptors such as TLRs. DCs process antigens into short peptides that associate with HLA molecules (Banchereau and Steinman, 1998). Secondly, DCs have the ability to mature and migrate via the lymphatics to secondary lymphoid organs where they encounter and provide stimulation for naïve T cells by serving as the main APCs (Banchereau and Steinman, 1998).

1.6.1 Identification and characterisation of DC subsets

Detailed analyses of DCs from various sites of the skin, thymus, spleen and BM have been instrumental in the recognition of multiple DC subsets, which are inducers of immunity or tolerance. In mice there are at least five distinct subsets, lymphoid DCs forming the predominant subset in the thymus and myeloid DCs, which dominate the spleen (Henri et al., 2001). The CD4 and CD8 T cell markers determine the DC subsets. The spleen consists of $CD4^-CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ DCs (Vremec et al., 2000). $CD8^+$ DCs are present in the T cell areas and $CD8^-$ DCs in the marginal zones, but they migrate to T cell zones when stimulated with microbial products (De Smedt et al., 1996; Reis e Sousa et al., 1997; Iwasaki and Kelsall, 2000). The $CD4^+CD8^+$ subset is dominant in the thymus. The double negative DC subset is present in all lymph nodes (LNs) and is thought to represent the mature DC.

In humans few studies have been performed on DCs freshly isolated from tissues due to the lack of sufficient numbers of cells obtained and the lack of specific markers for their identification. Therefore, the majority of information regarding human DC subsets, such as the origin and function, has been derived from *in vitro* studies using CB or BM $CD34^+$ haematopoietic stem cells (HSCs) (Young et al., 1995; Caux et al., 1996) or $CD14^+$ monocytes (Romani et al., 1994; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996; Randolph et al., 1998). Upon culture, $CD34^+$ HSCs or $CD14^+$ monocytes differentiate into DCs exhibiting high levels of expression of HLA Class II and costimulatory molecules. However,

based on the expression of membrane markers such as HLA-DR and CD11c, two DC subsets were originally identified in human peripheral blood (O'Doherty et al., 1994); the myeloid DCs (MDCs) and the plasmacytoid DCs (PDCs) (Grouard et al., 1997; Olweus et al., 1997; Dzionek et al., 2000). MDCs are $CD11c^{+}CD45RA^{-}$ $CD45RO^{+}$ and PDCs are $CD11c^{-}CD45RA^{+}CD45RO^{-}$ (O'Doherty et al., 1993). Both DC subsets lack expression of other haemopoietic lineage markers (lin^{-}), CD3 (for T cells), CD19 and CD20 (expressed by B cells), CD34 (HSC marker) and CD56 (NK cell marker). MDCs express CD11c, but the expression of IL-3 receptor (IL-3R) otherwise known as CD123, is low, whereas PDCs are negative for the CD11c marker and express high levels of CD123. There is a third minor subset of myeloid origin, which is $CD11c^{low}$ $CD123^{neg}$ (Dzionek et al., 2000).

Other, more recently identified markers include the blood dendritic cell antigen (BDCA)-1 also known as CD1c, which is expressed on MDCs (Dzionek et al., 2000) and B cells (Delia et al., 1988). BDCA3 (CD141) is expressed by the minor MDC population. BDCA4 is expressed by PDCs (Dzionek et al., 2000) and its expression is maintained following culture. BDCA4 is identical to neuropilin-1 (NP-1), a neuronal receptor for axon guidance factors belonging to the class 3 semaphorin subfamily (Kolodkin et al., 1997), and also a co-receptor for vascular endothelial growth factor (VEGF)-A on endothelial and tumour cells (Soker et al., 1998). The BDCA4 antigen was recently assigned the nomenclature of CD304 at the '8^{th} International Workshop on Human Leukocyte Differentiation Antigens' along with another PDC marker, BDCA2, which was assigned CD303. CD303 is a marker used to identify the PDC population by flow cytometry. It is a glycoprotein, which belongs to the C-type lectin family. CD303 differs from CD304 in that its expression is lost as PDCs mature in culture (Dzionek et al., 2000), and triggering by antibody potently inhibits type 1 IFN secretion (Dzionek et al., 2000; Dzionek et al., 2002)). Therefore, CD303 is not used to isolate PDCs from peripheral blood, and CD304 is exploited for this application. CD1c and CD304 can be used to isolate the two DC subsets separately in order to study their phenotype and function.

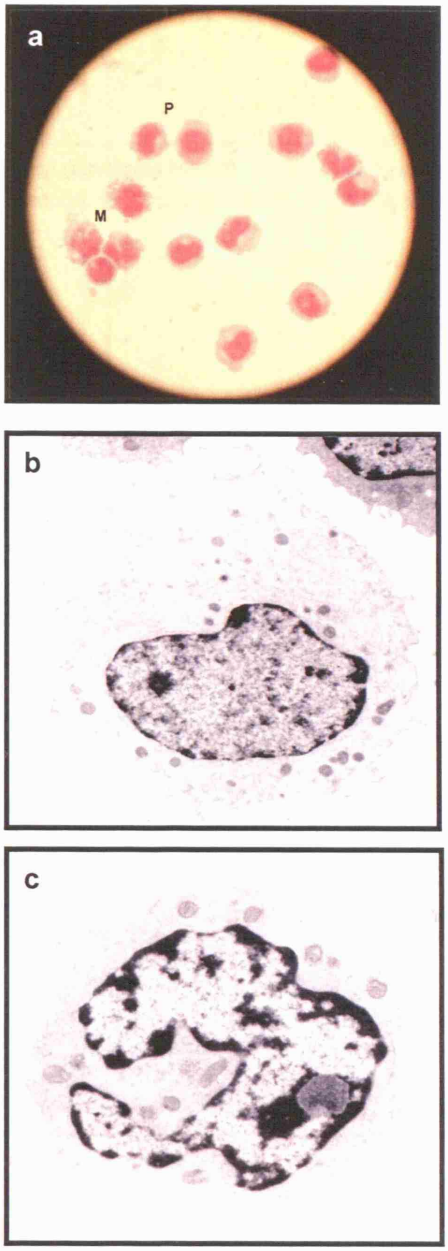
As well as differential marker expression, the cells are morphologically distinct. MDCs have a lobulated and heterochromatic nucleus, which occupies the majority

of the cell, and an irregular cytoplasm (Figure 1.4c). PDCs have a smaller, euchromatic rounded nucleus with abundant endoplasmic reticulum (ER) (Figure 1.4b) (Gomez et al., 2004). PDCs are associated with cells of the lymphoid lineage and referred to as plasmacytoid cells based on their plasma cell-like morphology (Siegal et al., 1999).

The majority of PDCs undergo apoptosis rapidly in culture but may be rescued by granulocyte monocyte-colony stimulating factor (GM-CSF) and IL-3 or culturing with a maturation stimulus such as CpG (Grouard et al., 1997). Under steady state conditions, peripheral blood PDCs reside in the blood and the T cell areas of lymphoid organs (LN and tonsils), unlike MDCs which are more widely located throughout the body (O'Doherty et al., 1994; Grouard et al., 1997; Olweus et al., 1997; Cella et al., 1999). MDCs are present in the blood, lymph, lymphoid tissues and within mucosal linings of the reproductive and gastrointestinal tracts.

1.6.2 The developmental origin of dendritic cells

The heterogeneity of DCs is indicated by the fact that DCs from different tissues possess differential morphology, phenotype and function and were initially thought to have been derived from distinct haematopoietic lineages. There are two proposed theories for DC development (Figure 1.5) (Shortman and Liu, 2002). The first is the 'functional plasticity model' where DC subsets are derived from a single lineage and the functional differences are a consequence of the effects of the local environment. This is based on a study showing a common DC precursor which generated the different DC subsets (del Hoyo et al., 2002). The second theory is of a 'specialised lineage model' in which different DC subsets are derived from early deviations in the developmental pathway, producing several distinct haematopoietic sublineages. This included a committed myeloid or lymphoid lineage (Grouard et al., 1997). A combination of both models may explain the variety and complexity of DC functions (Dakic and Wu, 2003). However, the origin and developmental pathways of DCs are still not yet completely understood. DCs were originally described as myeloid lineage derived cells due to their expression of early myeloid markers (CD13, CD33) (Inaba et al., 1993). A subsequent study showed that PDCs arose from lymphoid



(Gomez et al., 2004)

Figure 1.4. The morphological characteristics of dendritic cell subsets.

(a) Light microscopy of P (Plasmacytoid) and M (Myeloid) DCs (Giemsa stain x400). (b) Plasmacytoid and (c) Myeloid DCs ultrastructure obtained by electron microscopy (x7000). The individual DC subsets were examined under the electron microscope and images captured.

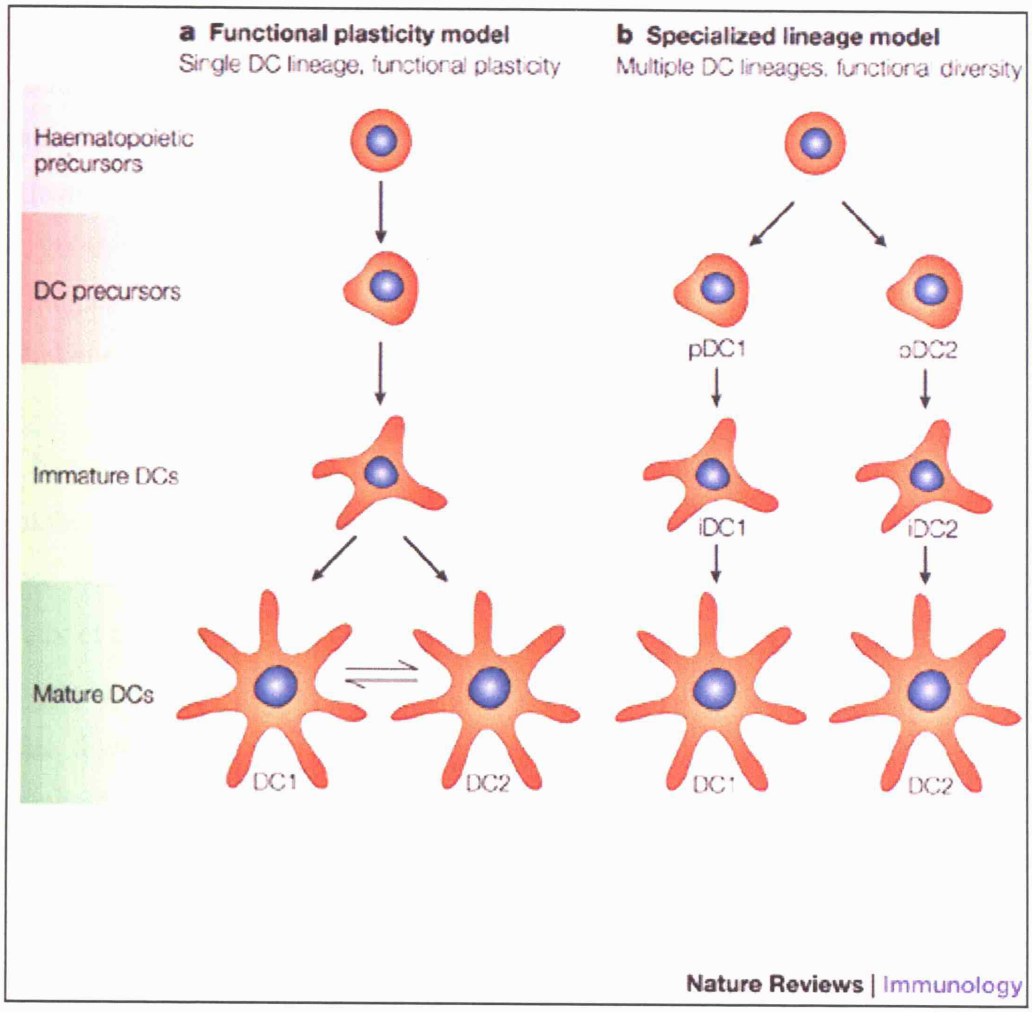


Figure 1.5. Alternative models for the generation of functionally distinct dendritic cells.

The two proposed models for the generation of different DC subsets include the (a) functional plasticity model placing emphasis on a single lineage from which DC subsets are derived, and (b) specialised lineage model in which separate lineages are established early in development (Shortman and Liu, 2002)

precursors (Wu et al., 1991; Galy et al., 1995; Kondo et al., 1997; Akashi et al., 2000). Theinitial evidence for DC subsets was described in mice by Akashi et al (Akashi et al., 2000). Mice DCs were defined as either of myeloid or lymphoid origin based on the expression of myeloid CD11b or lymphoid CD8α markers (Vremec et al., 1992). However, the requirement of different cytokines and involvement of different transcription factors indicates a divergent development pathway for the DC subsets (Saunders et al., 1996; Wu et al., 1998; Guerriero et al., 2000; O'Keeffe et al., 2003). Transferring to the human setting was difficult, as human DCs do not express CD8 a notable difference between the two species (Winkel et al., 1994).

In humans, DCs are derived from HSCs, but their origin can be myeloid or lymphoid. $CD34^+$ HSCs derived DCs that are $CD14^+$ represent blood DCs and those which are $CD14^-$ develop into $CD1a^+$ DCs resembling Langerhans cells (Caux et al., 1997). $CD34^+$ HSCs can also become lymphoid committed, driving the production of $CD11c^-$ DCs (PDCs) (Figure 1.6). The PDC subset can develop from thymic progenitors stimulated with IL-3 but not GM-CSF, and from lymphoid precursors in human tonsil treated with CD40L (Grouard et al., 1997; Shortman and Caux, 1997). Multipotential and lymphoid-committed precursors depend on Fms-like tyrosine kinase-3 ligand (Flt3-L) for PDC derivation (Blom et al., 2000; Spits et al., 2000; Blom et al., 2002; Briere et al., 2002; Schotte et al., 2003).

DC studies have also been performed using monocyte derived dendritic cells (MDDCs). MDDCs are derived from the culture of $CD14^+$ monocytes with a cocktail of cytokines, GM-CSF and IL-4 for 7 days, which drives the development of DCs, which are $CD14^-CD1a^+$ immature MDDCs. MDDCs are considered an "in vitro" model of their "in vivo" equivalents, MDCs. MDDCs are the most commonly studied source of DCs, due to their relative accessibility and abundant generation from monocytes. Availability of high numbers of MDDCs is not only an advantage for studying the characteristics of the DCs, but it is also important for the design and implementation of new therapeutic approaches such as vaccines for malignancies and for micro-organism infections. However, MDDCs are not

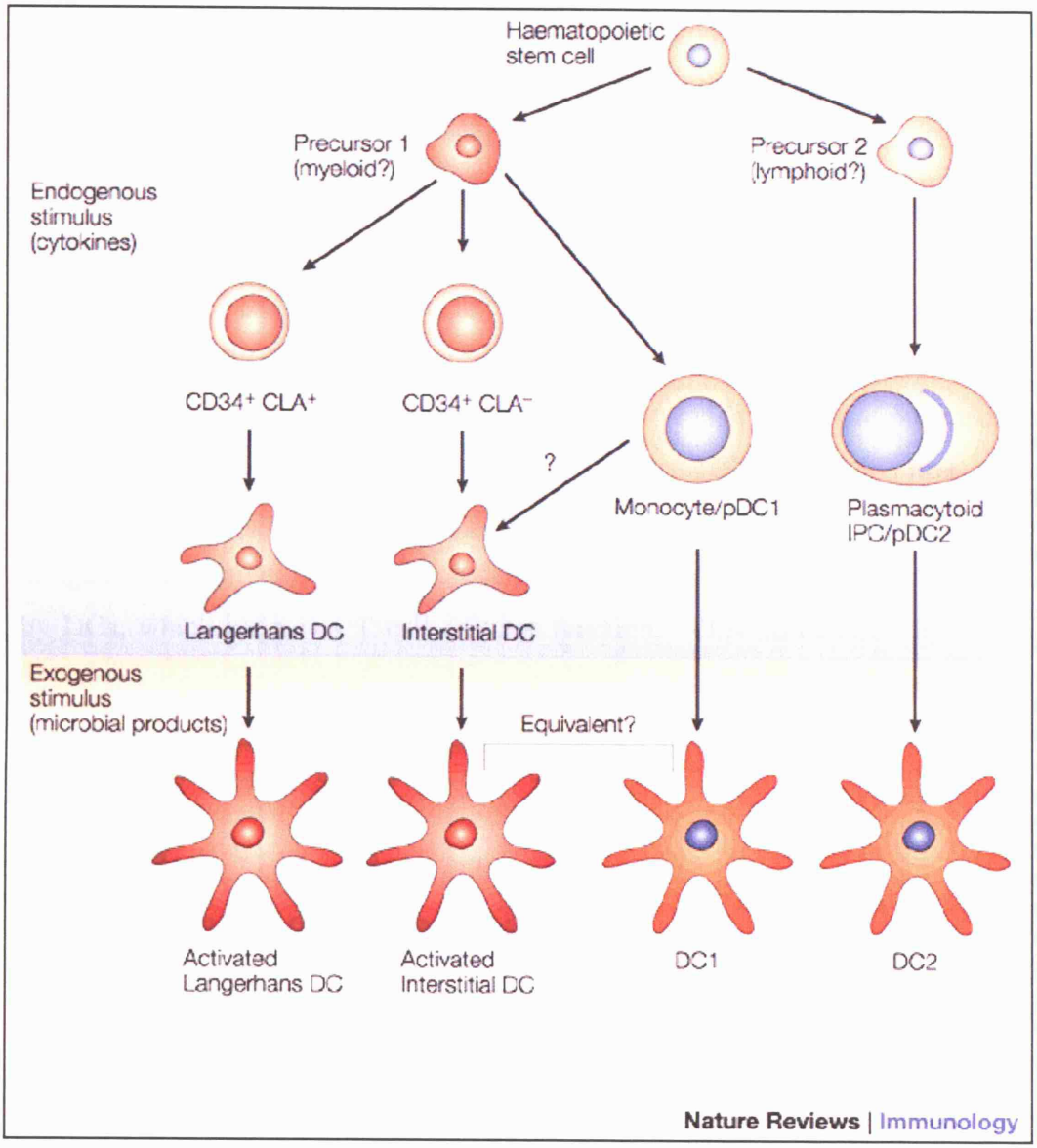


Figure 1.6. The developmental pathway of dendritic cells.

This is a schematic representation of the distinct variants of the human DCs, possible precursors and pathways of development (Shortman and Liu, 2002).

necessarily more effective than their alternatives (Mortarini et al., 1997; Osugi et al., 2002), although, they have been considered to mimic dermal type DCs (Sallusto and Lanzavecchia, 1994). Blood DCs represent a more physiological cell type not exposed to exogenous cytokines (Hart and Hill., 1997). MDCs are possibly precursors of MDDCs (or dermal DCs), since they migrate from the BM via the blood to the tissues of the body (Hart and Hill., 1997), where they differentiate into dermal DCs and LCs, both expressing HLA class II and CD1a molecules, but dermal DCs also express CD11b (Patterson et al., 2005).

1.6.3 Antigen recognition

Dendritic cells recognise antigen through a range of receptor-mediated mechanisms one of which involves TLR signalling. TLRs signal the presence of pathogens to DCs and are critical for DC activation. TLR signalling regulates antigen uptake (signal 1), costimulation (signal 2) and cytokine release (signal 3) by DCs, which promotes T cell effector function. TLR stimulation by PAMPs activates a signalling cascade involving proteins such as MyD88, further activating NF-κB (Figure 1.7), and induces the secretion of proinflammatory cytokines (TNFα, IL-1 and IL-12) that initiate an immune response, a pre-requisite to the activation of adaptive immunity. Ten TLRs (TLR1-10) have been described (Figure 1.7) and different combinations of them are expressed in different cell types reacting differentially to various microbial products. Distinct TLRs are specialised for different pathogens and their distribution varies with the type of DC (Kadowaki et al., 2001b). MDCs preferentially express TLR2, TLR3, TLR4, and TLR5, responding to synthetic microbial ligands such as PGN, lipoteichoic acid and LPS. The TLRs expressed by PDCs are restricted to those that recognise DNA and RNA of viruses. PDCs preferentially expressing TLR7 and TLR9, respond to synthetic CpG oligonucleotides (ODN) (Jarrossay et al., 2001; Kadowaki et al., 2001b; Ito et al., 2002). Although they have great plasticity, DCs are restricted in the microbes they can respond to, as they are reliant on their expression of TLRs. This difference may enable DC subsets to act alone or in concert initiating an immune response.

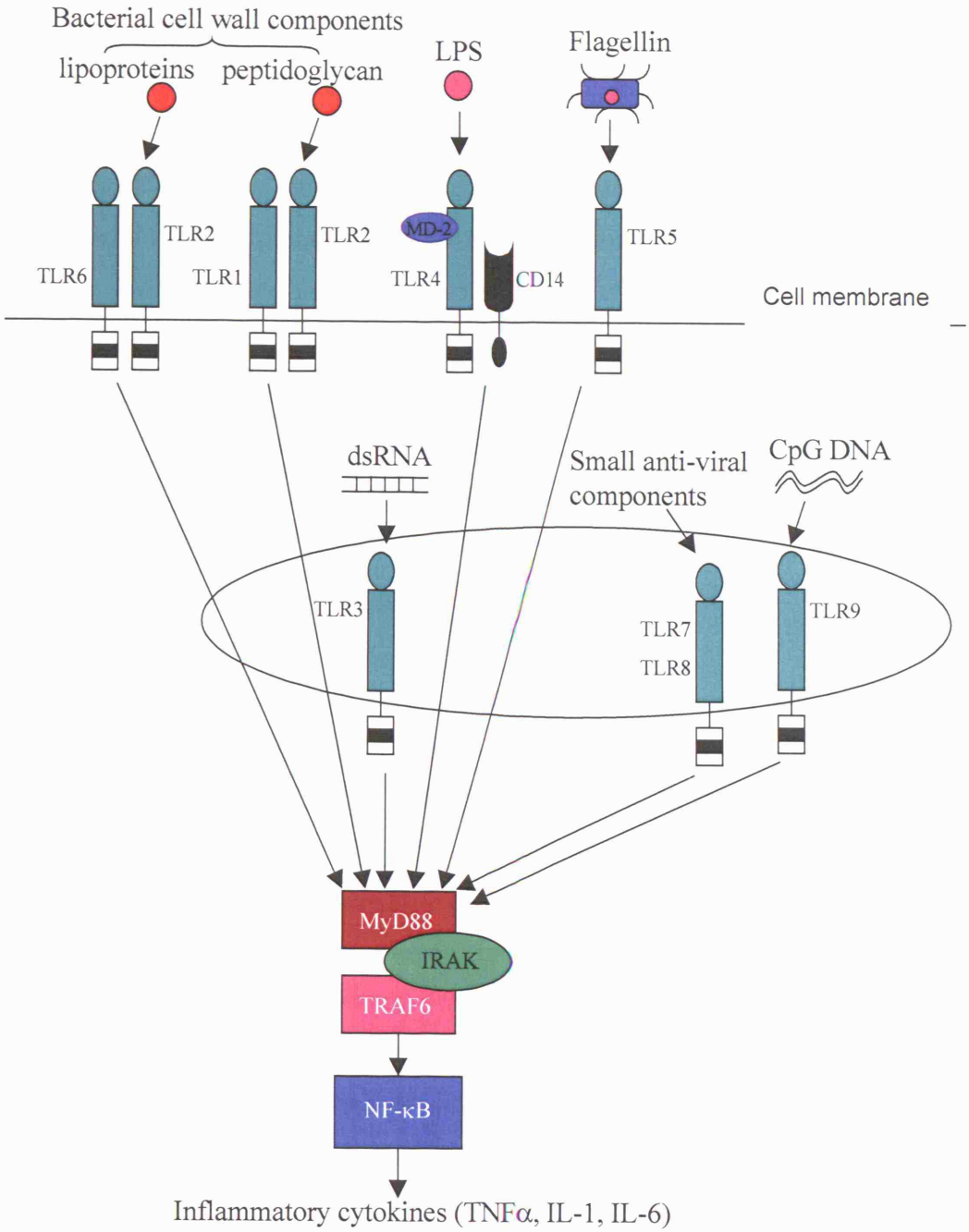


Figure 1.7. Toll-like receptor expression.

Ten members of the TLRs have been described so far, TLR1-10. They are differentially expressed on DCs and include TLR4 recognised by LPS and TLR9 recognising CpG. TLRs are specific for the microbe they encounter.

1.6.4 Antigen uptake

DCs are sentinels, assessing the environment for pathogenic material, which is taken up via endocytosis occurring in one of three ways, pinocytosis, receptor-mediated endocytosis or phagocytosis.

Non-specific soluble antigen uptake occurs through pinocytosis, which is subdivided into micropinocytosis, involving internalisation of small molecules (0.1 μm) via clathrin-coated pits, and macropinocytosis of larger molecules (0.5 - 3 μm) mediated by cytoskeletal membrane ruffling. Macropinocytosis has been established as a process applied in other cell types, but DCs can exclusively pinocytose exogenous antigen without signals from external stimuli (Sallusto and Lanzavecchia, 1994; Sallusto et al., 1995). Consequently, the engulfment of a large volume of fluid can be achieved, from which soluble antigens are concentrated for processing and presentation.

The second method of uptake, receptor-mediated endocytosis (Cella et al., 1997), may fall into two categories, either with the receptors directed to lysosomal compartments for degradation with examples of FcR, CD16 and CD32, or receptors targeted to early endosomal compartments for recycling to the cell surface and acting as a carbohydrate binding antigen uptake receptors (e.g. MR) (Sallusto et al., 1995; Avrameas et al., 1996) with differential expression on DC subsets (Banchereau and Steinman, 1998). The other lectin-type receptors, DEC-205 and DC-specific ICAM3 grabbing non-integrin (DC-SIGN) have also been described on DC subsets as endocytic receptors (Mahnke et al., 2000; Colmenares et al., 2002; Figdor et al., 2002; Cambi et al., 2003). The advantage of both macropinocytosis and receptor-mediated endocytosis is the efficient antigen presentation of very low concentrations of antigen.

Another antigen uptake method is phagocytosis, which involves ingestion of particulate antigens (Inaba et al., 1993). DCs are phagocytic cells but do not phagocytose to the same extent as other APCs, such as macrophages. These processes of antigen uptake function at maximum capacity in immature DCs, which express low levels of HLA class II and costimulatory molecules. Following

antigen uptake by one of the above mechanisms, the internalised material can then be processed.

1.6.5 Antigen processing

Following endocytosis, the antigen must be processed before presentation at the cell surface as an antigenic peptide. As previously stated, processing of antigen occurs through the HLA Class I and Class II pathways:

The HLA Class I pathway involves endogenous antigens, which are degraded in the cytosol by proteases and the peptides generated are transported into the ER by TAP (transporters associated with antigen processing), where they are loaded onto class I molecules and migrate to the cell surface via the Golgi network (Janeway et al., 2005). Although this pathway is used to produce peptides, the class II pathway is specific for APCs. However, DCs are unique in that they can process and present exogenous antigens through the HLA class I pathway (cross-presentation) (Yewdell et al., 1999).

The molecules associated with the HLA class II antigen processing pathway include HLA-DR, HLA-DM, Ii chain and CLIP (Class II-associated Ii chain peptide) (Figure 1.8). In this pathway, exogenous antigens are endocytosed by the cell and primed for processing by the HLA class II molecules. The HLA class II molecule complexes with the Ii chain and is transported from the ER to the endocytic compartment for binding peptide. The Ii chain is a non-polymorphic protein that binds the peptide binding groove of newly synthesised class II α/β heterodimers (Roche and Cresswell, 1990). It forms a scaffold to enable interaction with these heterodimers, therefore, acting as a chaperone. This prevents premature association with endogenous polypeptides. In immature DCs, the Ii chain is poorly degraded and the HLA class II molecules remain in the endocytic compartments. Once mature, following Ii chain degradation by enzymes in the late endosomal compartment, most HLA class II molecules are present at the cell surface of DCs. Therefore, the Ii chain also regulates intracellular trafficking of HLA class II dimers. Following degradation, the residual fragment of the Ii chain, CLIP, remains, occupying the groove. CLIP

binds the peptide-binding groove of the HLA class II molecule to prevent premature peptide binding (Ghosh et al., 1995; Shi et al., 2000; Stumptner-Cuvelette and Benaroch, 2002). To remove CLIP, another chaperone molecule, HLA-DM (or H-2M in mice) is recruited, catalysing the release of CLIP and allowing a peptide to bind, in turn enabling the DC to present peptide at its cell surface (Figure 1.8) (Morris et al., 1994; Denzin and Cresswell, 1995).

Another antigen processing system involving DCs consists of the CD1 family of molecules (Porcelli and Modlin, 1999). The CD1 molecules are nonpolymorphic histocompatibility antigens associated with β_2M (Brenner and Porcelli, 1997; Shinkai and Locksley, 2000). CD1 molecules are important in presenting non-protein lipid and glycolipid antigens derived endogenously or exogenously. The molecules are expressed on the cell surface following transport via vesicles. They are subsequently incorporated into endosomes associating with lipid ligands and recycle back to the cell surface. Depending on the CD1 homologue, an antigen associates in distinct endosomes (Sugita et al., 1999). CD1a binds to lipid antigens only in early endosomes. CD1b binds to lipids degraded in late endosomes, whereas CD1c and CD1d associate with their corresponding lipid antigens in early endosomes. CD1 antigen presentation occurs before Class II antigen presentation.

1.6.6 Antigen presentation

Following encounter of pathogen, uptake and processing of antigen, DCs become specialised in antigen presentation for T cell activation. DCs upregulate HLA, adhesion and costimulatory molecule expression. As established, DCs are the vehicles of immunity following exposure to foreign antigens, and they also determine the polarisation of Th responses after arriving in the draining LNs, which is the major site of DC-T cell interactions. A Th1 immune response is mounted to remove viruses and intracellular bacteria, involved in cell mediated immunity. The cytokines IL-2 and IFNγ favour the development of Th1 cells (Banchereau and Steinman, 1998; Tarte and Klein, 1999). A Th2 immune response is associated with humoral immunity and clears parasitic infections or dampens excessive Th1 immunity that may cause damage or autoimmunity. Th2

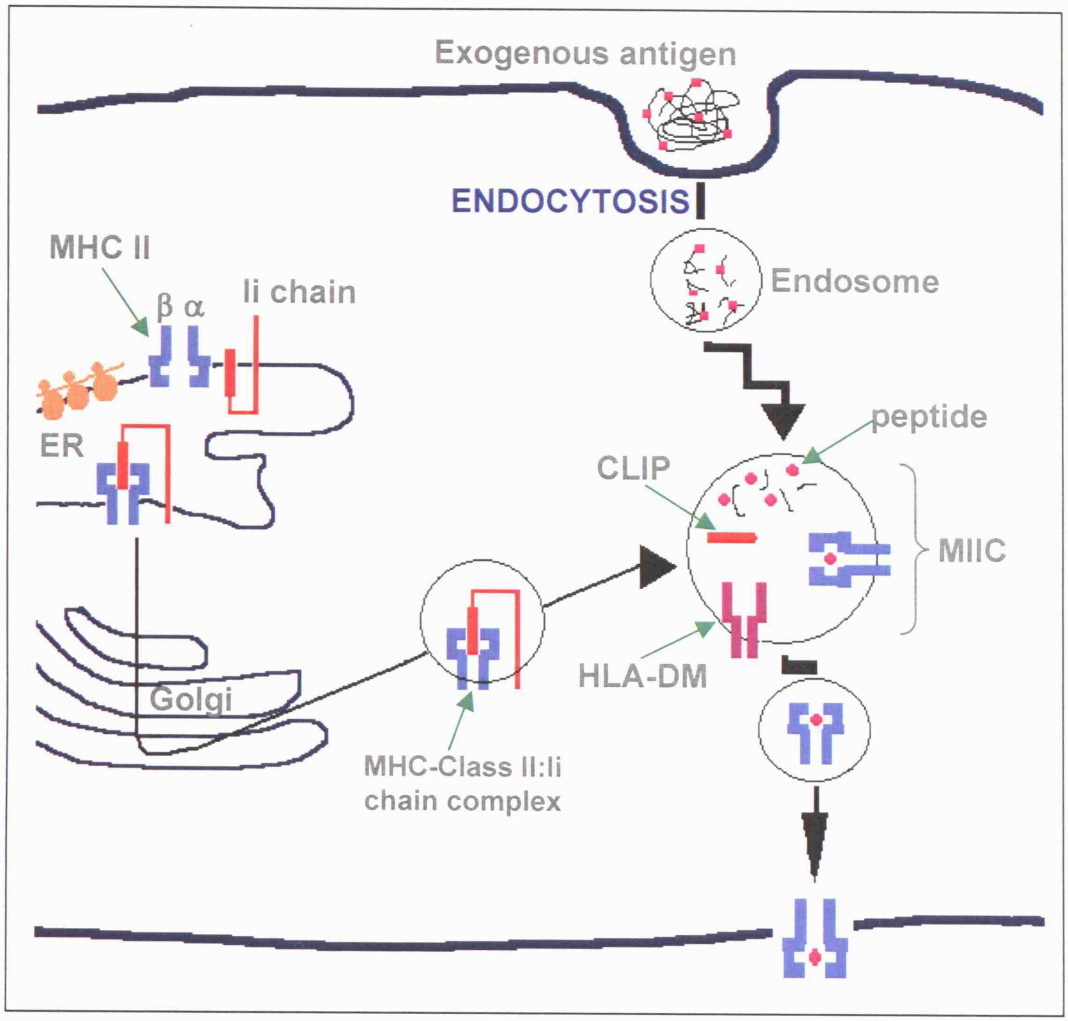


Figure 1.8. The MHC-II endocytic antigen processing pathway.

This pathway involves endocytosis of exogenous antigen by the cell and involvement of the various antigen processing molecules, HLA-DR, HLA-DM, Ii chain and CLIP. Once the antigen is processed is can be presented at the cell surface to $CD4^+$ T cells.

cells are identified by the secretion of IL-4, IL-5, IL-10 and IL-13 (Abbas et al., 1996). The type of cells generated also change over the course of the response. Initially, large numbers of effector cells are generated to clear the infection. This is a result of the production of IL-12, which induces the secretion of IL-2 and IFNγ (Heufler et al., 1996). Later, memory cells are established to protect against a subsequent infection by the same pathogen. In this instance, IL-4 secreting T cells are generated to help dampen the Th1 response (O'Garra, 1998). T cell polarisation can be determined by the stimulus encountered, cytokine environment at priming, the nature and intensity of TCR mediated activation signals, costimulatory signals, the type of DC-antigen presented to naïve T cells, DC-T cell ratio, DC origin and the genetic background of T cell donors. T cell responses depend on the duration and intensity of the interaction of the DC with the T cell, but this is overcome by the influence of the cytokine profile and effector function of the Th directed cells.

It was proposed that different developmental lineages of DCs could induce Th1 or Th2 responses (Shortman and Liu, 2002). Therefore, functionally, the two DC subsets differ, with MDCs being immunogenic and inducing an IL-12-dependent Th1 immune response and PDCs derived from $IL3R\alpha^{+}CD4^{+}CD3^{-}CD11c^{-}$ plasmacytoid precursor cells drive an IL-4-independent Th2 response (Rissoan et al., 1999; Liu et al., 2000). The differential Th cell polarisation is not as simple as first thought and has been controversial. Recent reports have demonstrated polarisation towards either Th cell development by both DC subsets, depending on the cytokines and stimuli received (Cella et al., 2000; Ruedl et al., 2000; Boonstra et al., 2003). MDCs can induce tolerance when immature (Kelleher et al., 1999; Reid et al., 2000) and PDCs direct cells toward a Th1 cell repertoire with the appropriate stimulus (Maldonado-Lopez et al., 1999; Pulendran et al., 1999; Cella et al., 2000). It has also been shown recently that PDCs in combination with limited numbers of MDCs can induce a Th1 immune response (Naranjo-Gomez et al., 2005). The PDC population primarily functions as a modulator of anti-viral responses, but its antigen presenting role is initiated when necessary. This flexibility of DCs priming Th responses may contribute to the simultaneous generation of various Th cells. There is also evidence that DCs can induce T_{regs}

through the production of IL-10 (Gilliet and Liu, 2002; Wakkach et al., 2003; Jonuleit et al., 2000).

1.6.7 Dendritic cell maturation

On contact with inflammatory mediators, DCs undergo maturation where their role changes from antigen capturing and processing to antigen presentation. DC maturation is a multi-level process, comprised of morphological, immunophenotypic and functional changes corresponding to DC differentiation pathways and migratory capacity, antigen uptake and presentation capacity and potential to induce cytokine production, immunity or tolerance. DCs display different stages of maturation which encompass precursors in the BM and blood, immature DCs in tissue and mature DCs within secondary lymphoid organs. Immature DCs are small cells similar in appearance to lymphocytes. On maturation they develop dendrites, which extend to the surrounding environment, facilitating interactions with T cells. The different stages of DC maturation affect the function of these cells and various stages of maturation have been described; immature, semi-mature, or fully mature (Figure 1.9) (Lutz and Schuler, 2002; Shortman and Liu, 2002). Immature DCs have low expression of HLA class II and costimulatory molecules, do not secrete cytokines, but are highly endocytic. Upon stimulation with factors such as LPS or antigen, the cells undergo maturation resulting in an upregulation of HLA class II and costimulatory molecule expression, and cytokine production is initiated. DCs secrete various cytokines including IL-12, TNFα, IL-1 and IL-6 (Saint-Vis et al., 1998; Lutz and Schuler, 2002). The two DC subsets preferentially secrete cytokine upon stimulation. MDCs produce IL-12 whereas PDCs are regularly referred to as IFN-α-producing cells (Cella et al., 1996; Heufler et al., 1996; Cella et al., 2000). When DCs are semi-mature, the secretion of cytokines is limited to IL-10 secretion and these DCs are thought to induce tolerogenic T cells (Kourilsky and Truffa-Bachi, 2001). Their endocytic capacity is diminished and DCs are no longer able to process antigen but become efficient in presenting processed antigenic peptides to T cells. The ability of individual DC subsets to induce tolerance or immunity may be due to the fact that they interpret the environment differently and signal to T cells accordingly.

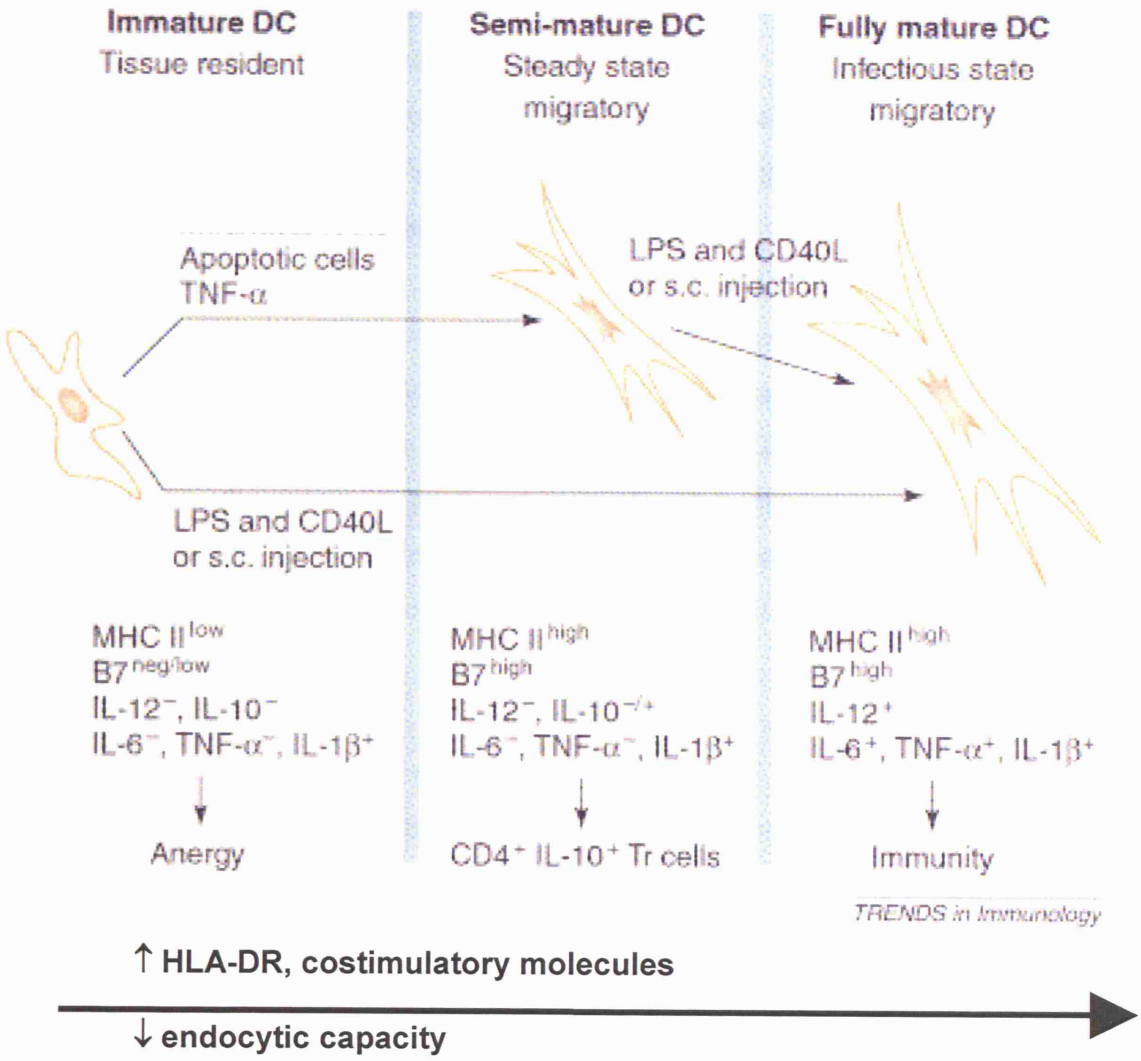


Figure 1.9. The maturation stages of dendritic cells.

The different stages of DC maturation include the immature DC, the semi-mature DC and the fully mature DC (Lutz and Schuler, 2002).

1.6.7.1 Secretion of cytokines by dendritic cells

Dendritic cell maturation induces secretion of various cytokines, which are soluble proteins that regulate cellular activity within the immune system. Cytokines play an important role in modulating immune responses following binding to specific cell surface receptors which signal to their target cells. Cytokines have diverse biological effects and interleukins and colony stimulating factors (CSFs) are examples of mediators which have a direct effect on cell proliferation, while IFNs are cell growth inhibitory factors (Theze, 1999).

Cytokines act at very low concentrations and are short-lived. They may act locally (autocrine, paracrine) or at a distance (endocrine) following release. DCs produce various cytokines in response to a range of antigens (Zhou and Tedder, 1995). Depending on the cytokines produced by a DC, the responding T cells either ignore the antigen, induce tolerance or mount an immune response. The various cytokines secreted by cells include those denoted proinflammatory, Th1 and Th2 cytokines.

1.6.7.1.1 Proinflammatory cytokines

Proinflammatory cytokines are critical in early responses to pathogens and upregulation of local host defences. They are also involved in the pathogenesis of Graft versus Host Disease (GvHD) (Holler, 2002; Schmaltz et al., 2003). Examples of proinflammatory cytokines include TNFα, IL-1β and IL-6. TNFα is one of the most pleiotropic cytokines affecting cells by altering their growth, differentiation and survival patterns. TNFα is critical to the survival of infected DCs, controlling intracellular pathogen growth. In addition to its capacity as an inducer of DC maturation it may have an autocrine or paracrine effect during infection, enabling DCs to bridge the gap between innate and adaptive immunity. Receptor-ligand interaction between various TNFRs and their corresponding ligands influences both DC maturation and T cell priming. The spectrum of action of IL-1β is similar to TNFα, and to a lesser degree, IL-6. Unlike TNFα, IL-1β does not kill cells and is a BM stimulant that increases the number of myeloid progenitor cells (Theze, 1999). IL-6 regulates immune reactivity, inflammation and haematopoiesis. Its release is stimulated by TNFα and IL-1β, but IL-6

persists in the plasma for longer periods than the other proinflammatory cytokines (Theze, 1999). The secreted TNFα, IL-1β and IL-6 are involved in regulating T cell growth and differentiation (Suda et al., 1990).

1.6.7.1.2 Th1 cytokines

Dendritic cell subsets secrete several cytokines, but the two main Th1 cytokines produced by MDCs and PDCs are IL-12 and IFNα, respectively. The ability of DCs to produce IL-12 depends on their origin, is increased by an anti-inflammatory environment and occurs within a few hours of infection (bacteria and intracellular parasites) (Macatonia et al., 1995). IL-12p70 is the bioactive heterodimer of the 35kD (L chain, p35) and 40kD (H chain, p40) disulphide linked subunits encoded by two separate genes (Trinchieri, 1998). The importance of IL-12 as an IFNγ inducer is its high efficiency at low concentrations and synergy with other activating stimuli. A combination of IL-12 and CD80/86 expression induces efficient T cell proliferation and cytokine production (Liu et al., 2001b). Another Th1 cytokine, IFNα, is a member of the family of type 1 IFNs. Type 1 IFNs are important in antiviral immunity and are produced by several cell types including macrophages, fibroblasts and MDCs, but the cell which is a potent producer of IFNα is the PDC. Removal of this DC subset has resulted in the reduction of type 1 IFN production (Asselin-Paturel et al., 2005). By contrast, overproduction can result in the development of autoimmune diseases such as SLE (Palucka et al., 2005). Therefore, the production of IL-12 and type I IFNs (e.g. IFNα) that induce Th1 differentiation suggest a connection between the innate and adaptive immune response, effective when various pathogens are encountered.

1.6.7.1.3 Th2 cytokines

IL-4, IL-10 and IL-13 are all examples of Th2 cytokines. IL-4 positively drives Th2 differentiation, promotes myeloid DC differentiation when cultured with GM-CSF and primes the production of IL-12 by MDCs (Moser and Murphy, 2000). IL-4 and IL-13 are the predominant Th2 cytokines produced in allergic diseases. Blocking IL-13Rα1 chain or IL-4R prevents apoptosis restoring the Th1 response.

IL-10 is a regulatory cytokine inhibiting Th1 immune responses, potently suppressing the proliferative and cytolytic responses, which involves interaction between DCs and T cells by downregulating HLA Class II expression (Buelens et al., 1995). The ability of IL-10 to act as an anti-inflammatory soluble factor emerged from studies where it inhibited proinflammatory cytokines particularly those produced by the monocytic lineage (IL-1β, IL-6, TNFα, GM-CSF) (de Waal et al., 1991). Endogenous production of IL-10 is associated with allograft acceptance and neonatal tolerance (VanBuskirk et al., 2000; Rainsford and Reen, 2002). However, high doses of exogenous IL-10 can be detrimental to the prognosis of GvHD and possibly lethal (Blazar et al., 1998). Therefore, it is essential to monitor levels of exogenously administered cytokines.

1.7 Neonatal immunity

BM and PBSCs used in transplants are foreign material used to treat diseases. However, the fetus is a foreign tissue that is consistently grafted and tolerated by the maternal environment. This is remarkable as the fetus carries paternal MHC and minor histocompatibility antigens that differ from the mother and would be expected to be rejected. The placenta is a tissue that directs the fetus away from maternal T cells. The outer layer of the placenta consists of the trophoblast, which forms the feto-maternal interface and lacks classical HLA molecule expression (Pazmany et al., 1996). The trophoblast is, therefore, resistant to recognition and attack by maternal T cells. The trophoblast does, however, express the nonclassical HLA class I molecule, HLA-G, which is known to bind inhibitory NK receptors, KIR1 and KIR2 and is therefore, protected from attack by NK cells (Carosella et al., 1999; Schust et al., 1999).

Another factor contributing to maternal tolerance of the fetus is cytokine secretion at the feto-maternal interface. The feto-maternal interface exhibits a Th2 cytokine pattern (IL-4, IL-10 and TGFβ) throughout gestation (Lin et al., 1993). The secretion of Th1 cytokines such as IFNγ and IL-12 induce spontaneous abortion (Rice and Chard, 1998). Therefore, the fetus may be regarded as an immunologically privileged site, protected by a tissue barrier and

immunosuppressive responses. This suggests that the fetus and thereafter the neonate, possess characteristics which differ from adults. Therefore, tolerance to the fetus may hold the key to inducing specific tolerance to grafted tissues.

Newborns have an increased sensitivity to infectious agents, which may be due to the lack of pre-existing immunological memory (Siegrist, 2001) and the small number of immune cells in peripheral lymphoid tissues (Lu et al., 1979; Lu et al., 1980; Levin and Gershon, 1989; Siegrist, 2001). Therefore, they fail to mount protective responses against several pathogens. The functional impairment of neonates may result from the combined effects of true cellular immaturity, naivety or lack of previous exposure to antigens, and intra-uterine exposure to hormones (progesterone) and cytokines (IL-4), which may favour Th2 development (Piccinni et al., 1995; Piccinni and Romagnani, 1996). These deficits prompted studies to define the immunologic features of cord blood (CB) cells (Wagner et al., 1995; Cairo and Wagner, 1997). CB immune cells are quantitatively and qualitatively distinct from adult cells. Subsets of cells are present in different proportions in neonates and adults and those of the same subset have phenotypic differences. For example, CB T cells are naïve in nature expressing specific surface markers (CD45RA). By contrast, the adult T cell population possess markers of both naivety (CD45RA) and memory (CD45RO). The T cells in CB were initially described as having a naïve phenotype, $CD45RA^{+}/CD45RO^{-}$, $CD62L^{+}$ and impaired functional capabilities compared to APB (Chalmers et al., 1998; Szabolcs et al., 2003b). Neonatal immune cells were originally considered as immature, but in 1996, several studies showed that neonates could mount adult level T cell responses to infections (Forsthuber et al., 1996; Ridge et al., 1996; Sarzotti et al., 1996). Therefore, under appropriate conditions of stimulation, neonatal cells can fight intracellular pathogens.

The generally accepted rule has been that the naivety of CB T cells is the major factor contributing to the reduced incidence and severity of GvHD after CBT (Chalmers et al., 1998; Cohen and Madrigal, 1998). This has raised questions over the properties of the neonatal immune system, which may account for the reduction. It is now thought that other cell types are involved. NK cells have also been studied and these are phenotypically distinct between CB and APB (Read and

Williams, 1984). It was, therefore, hypothesised that DCs, the major players of the immune response, could have divergent functions in CB and APB.

1.7.1 Cord blood dendritic cells

The phenotype and function of the two DC subsets in CB are not well established. PDCs were the first DC population to be identified in CB (Sorg et al., 1999), followed by the myeloid subset (Borras et al., 2001). In APB, there is a 60:40 ratio of MDC:PDC, although in CB there are several contradictory reports, some suggesting an inverted ratio of MDC:PDC (40:60) (Sorg et al., 1999; Borras et al., 2001) and others suggesting ratios similar to those found in APB (Schibler et al., 2002; Darmochwal-Kolarz et al., 2004). Other differences include those of phenotype and function, with CB DCs expressing lower levels of surface molecules including HLA-DR and displaying reduced allostimulatory capacity (Liu et al., 2001a).

These initial differences prompt the further study of the two DC subsets isolated from CB and comparison with the results from APB. The findings would be important in understanding the immune response in the newborn compared to the adult population. This may help explain the reduced GvHD observed following umbilical cord blood transplantation (CBT) in comparison to bone marrow transplantation (BMT).

1.8 Haematopoietic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is undertaken in patients with haematological and immunological disorders such leukaemia, as their own immune system is weak or non-functioning following chemotherapy due to killing of cancerous and healthy cells. It is the intravenous infusion of HSCs to replace BM function in a patient with a defective BM (Tabbara, 1996b; Tabbara, 1996a; Ikehara, 1998). There are several sources of stem cells including BM, PBSCs and CB. All three have advantages and disadvantages, but are used to achieve one goal, to treat life-threatening conditions.

1.8.1 Bone marrow transplantation

Allogeneic BMT has been the main therapeutic procedure for stem cell reconstitution following treatment for a variety of haematological malignancies such as chronic myeloid leukaemia (CML) and acute lymphoblastic leukaemia (ALL) (Ikehara, 1999), and genetic disorders (Tabbara, 1996a) such as Fanconi's anaemia. The conditioning regimen involves chemotherapy and irradiation to completely ablate the patient's BM followed by replacement with HSCs from a selected donor.

There are several advantages of BMT over CBT, which include rapid engraftment, availability of a large stem cell dose, reduced risk of transplanting a genetic disease and the donor may be available for a second transplant or to provide T cells. However, the disadvantages preventing successful engraftment include risk of opportunistic infections such as Cytomegalovirus (CMV) and Epstein Barr Virus (EBV), low proliferative potential and lengthy search times (can take several months). BMT is an invasive procedure and the donation requires surgery under general anaesthesia. A major limiting factor is that of finding a HLA matched donor, which is crucial for successful engraftment. When BM cells are used as a therapy to restore immune function, the graft provides the HSCs, T cells and APCs. If the HLA types are disparate, an allogeneic response is mounted. Hence, the ideal donor for BMT would be an HLA-identical twin or sibling. As numbers of these are limited, HLA-matched allogeneic unrelated donors are frequently used as an alternative BM source. Unfortunately, allogeneic BMT is associated with serious side effects, most commonly GvHD. GvHD may result when the mature T cells in the graft BM recognise the host as foreign and mount an immune response. Various strategies have been adopted to reduce GvHD including T cell depletion of the graft (Link, 1999). This is, however, accompanied by a reduction in the beneficial graft versus leukaemia (GvL) effect (Apperley et al., 1986; Maraninchi et al., 1987; Marmont et al., 1991).

Although transplantation is a life-saving therapy, the deleterious effects including acute rejection, long-term graft loss and GvHD, and infectious complications have limited its beneficial potential. Due to these complications, various alternatives

were investigated, which included gene therapy, peripheral blood stem cell transplantation (PBSCT) and CBT (Arcese et al., 1998). All cell sources are likely to have advantages and disadvantages, but CBT has been suggested to be associated with a lower degree of GvHD (Madrigal et al., 1997). As GvHD is a major cause of mortality following transplantation, CB is an alternative source of HSCs for patients requiring haematopoietic reconstitution.

1.8.2 Cord blood transplantation

Cord blood was initially identified as a stem cell source in the 1960s. However, it was not until 1988 that CB was used in the transplantation setting to treat a patient with Fanconi's anaemia (Gluckman et al., 1989). By the 1990s CB banks were being established world-wide (U.K., U.S.A, France, Italy), introducing protocols for collection, processing and freezing of CB units (Rubinstein et al., 1995). To date, over 4000 transplants using CB have been performed worldwide (Ballen, 2005).

Cord blood is used as an alternative source of stem cells due to its immediate availability as it is a waste product and there is no risk to the donor or donor attrition. There is a reduced risk of transmissible infectious diseases (CMV, EBV) compared to BMT, as they do not traverse the feto-maternal barrier (Varadi et al., 1995). CB contains a significantly higher number of early and committed progenitors with a higher proliferative potential than BM (Payne et al., 1995). An important factor, which is absent in BM registries, is the broad representation of ethnic groups, a result of CB banks targeting those hospitals with a large ethnic minority intake. Therefore, there is a greater ability to expand the available donor pool targeting ethnic minorities. CB units can be cryopreserved and, therefore, are available on demand within weeks instead of months, which is the length of time a BM search can take. Another major advantage of CB is that a degree of HLA incompatibility can be tolerated (Wagner et al., 1996; Barker et al., 2001). Therefore, a lower incidence and severity of GvHD has been observed in related transplants in children (Rocha et al., 2000). For unrelated donor transplantation, data suggested that engraftment occurred even with HLA mismatches, with a surprisingly low risk of GvHD (Gluckman et al., 1997). This increases the

potential donor pool as compared with either BM or PBSCs. However, a higher cell dose, particularly, a high CD34 count, was an important prognostic factor, improving engraftment and survival (Wagner et al., 2002). CB has also been successfully used to treat adult patients with malignancies such as leukaemia and lymphoma (Laughlin et al., 2001).

The disadvantages of transplanting CB include the risk of transmission of genetic disorders (Ballen, 2005). The volume obtained is limited, in turn, limiting the stem cell dose, possibly a factor delaying engraftment. However, this is being overcome by transplanting two CB units resulting in successful engraftment (Wang et al., 2005). Although there is no known definitive explanation for the advantage of CB over other stem cell sources, the reduced incidence and severity of GvHD following transplantation is a major finding that encourages its use.

1.9 Graft versus host disease

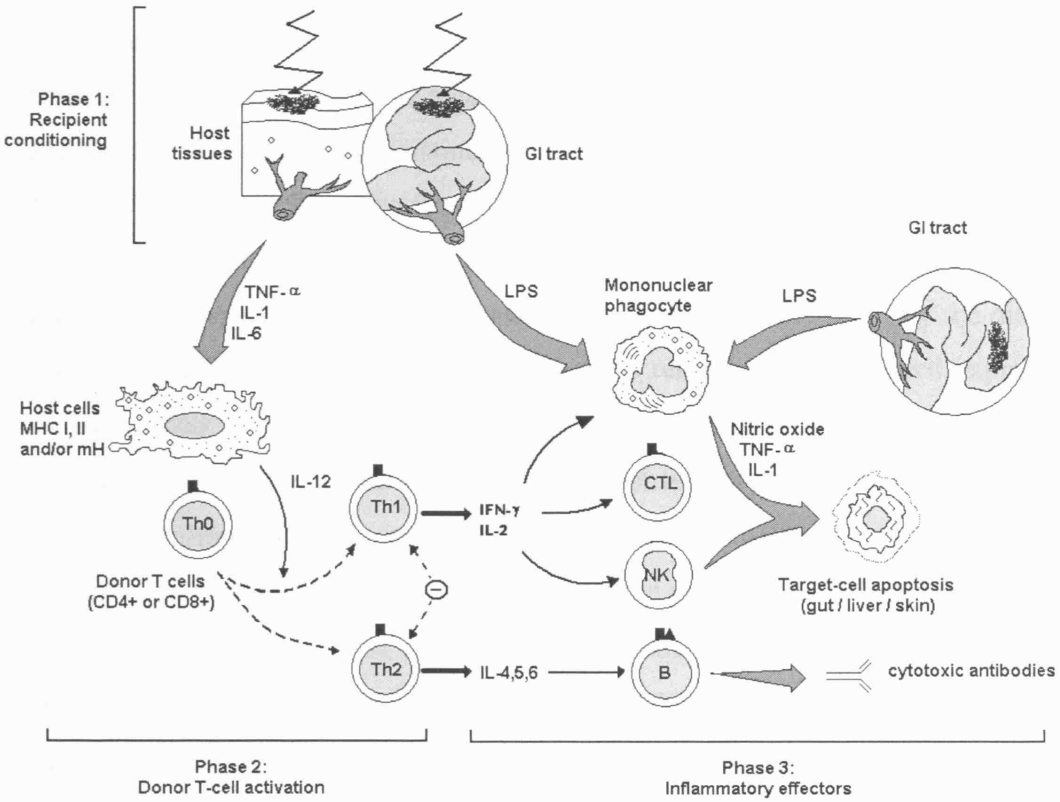
GvHD is a consequence of immunocompetent cells from the graft being introduced and recognising alloantigens present in the immunologically incompetent recipient. This results in multi-organ system dysfunction and destruction. The main cause of GvHD is HSCT where there is presentation of histoincompatible recipient antigens to donor T cells (Murphy and Blazar, 1999). Solid organ transplants, blood transfusion and feto-maternal transfusion have also reportedly caused GvHD (Rappeport, 1990). Criteria for the development of GvHD, described in the 1960s included the fact that the graft must contain immunocompetent cells, the host must appear foreign to the graft, and the host must be incapable of reacting sufficiently against the graft (Billingham, 1966).

There are two forms of GvHD, the first being acute (*a*GvHD), which occurs within the first 100 days of transplant presenting with redness of the skin 10–30 days post transplant (Ferrara and Deeg, 1991; Ferrara et al., 1996). As the disease progresses there is more skin, gut and liver involvement and this gives rise to incidences of dermatitis, enteritis and hepatitis, which are graded I-IV. This form of GvHD is a major complication of allogeneic BMT and is the major cause of

death in up to 40% of patients, due to the progression to sepsis. The second form, chronic GvHD (*c*GvHD), develops after day 100 post-transplant presenting with an autoimmune syndrome, which is directed towards multiple organs (Atkinson, 1990; Perez-Simon et al., 2006). The skin is the primary organ involved, but there is also oral and ocular involvement. Chronic GvHD may occur as a late phase of *a* GvHD in 70-90% of patients or as a distinct entity. The likelihood of *c*GvHD increases with the severity of *a*GvHD. Patients with grade III or IV *a*GvHD are more likely to develop the chronic form. The rate of mortality is dependent on the mode of development, with the highest rate of mortality resulting from progression of acute to chronic.

The extent of histoincompatibility between the donor and host and the residual number of T cells in the graft are major criteria affecting the incidence of GvHD. No single cell type has yet been identified as the main effector of GvHD. Both NK cells and T cells have been involved in the initial inflammatory response (Bryson and Flanagan, 2000). In the allogeneic setting, immunosuppression and T cell depletion are procedures used to prevent GvHD, but can also result in the failure of the graft, increased incidence of leukaemic relapse and loss of the GvL effect (Maraninchi et al., 1987; Marmont et al., 1991).

The various cell sources used in transplants may influence the outcome of GvHD. The incidence of GvHD increases when allogeneic PBSCs are used rather than autologous cells and peaks when BM is used as a HSC source. The recent use of CB as a stem cell source appears to reduce the risk of GvHD development, but there is delayed haematopoietic recovery (Rocha et al., 2001). It is thought that the immunologic immaturity of CB cells may be influential in the suggested outcome of GvHD.



Modified from: G R Hill et al. Cytokines, Cellular and Molecular Therapy \ 1997 \ Volume 3

Figure 1.10. Cytokine dysregulation in graft versus host disease.

The three sequential phases in the development of GvHD include the afferent phase of host conditioning (Phase 1), the amplification phase where there is donor T cell activation (Phase 2) and the efferent phase involving inflammatory effectors (Phase 3) (Hill et al., 1997).

1.9.1 Cytokines involved in the process of GvHD

Although the initial development of GvHD involves contact between the donor T cells and host APCs, interactions between immunocompetent cells are further governed by cytokines. Studies have examined the roles of the various cytokines in the pathogenesis of GvHD. Current evidence suggests that dysregulated cytokine production occurs as a cascade of events during cell activation (Krenger and Ferrara, 1996a). Three distinct phases have been described in GvHD (Figure 1.10) (Hill et al., 1997). The first is the afferent phase (Phase 1) which is initiated by the direct recognition of recipient alloantigens by donor T cells which are infused into the recipient whose immune system has been damaged by the disease, infection and/or the conditioning regimen. This results in an increased expression of HLA class II and adhesion molecules and the release of proinflammmatory cytokines (TNFα, IL-1, and IL-6) from the host tissues. The amplification phase (Phase 2) follows, where interaction of donor T cells with recipient APCs results in activation and expansion of T cells driving the polarisation of Th1 or Th2 cells, and subsequent secretion of cytokines. Cytokines such as IL-2, TNFα and IFNγ enhance T cell expansion, induce CTL and NK cell responses and prime the further production of TNFα and IL-2. These proinflammatory cytokines stimulate chemokine production, enabling recruitment of effector cells to target organs. The efferent phase (Phase 3) consists of a complex cascade of multiple effectors. The effector functions of mononuclear phagocytes are triggered by a secondary signal provided by LPS that leaks through the intestinal mucosa damaged during Phase 1. This may amplify local tissue injury. The cytokines produced by these cells activate inflammatory effector cells (CTL and NK) that result in direct or indirect (by nitric oxide, TNFα, and IL-1) host tissue damage. A balance between Th1 and Th2 may be essential in controlling the development of GvHD (Krenger and Ferrara, 1996b). Therefore, the cytokines secreted in response to an experimental GvH effect may help to understand the outcomes of CBT and BMT.

1.9.2 Dendritic cells and GvHD

There are two proposed mechanisms for the induction of GvHD, direct and indirect allorecognition (Figure 1.11). Direct allorecognition is well established as the predominant mechanism of GvHD initiation. It involves donor T cells recognising recipient alloantigen on recipient DCs (Shlomchik et al., 1999). The indirect pathway involves donor DCs presenting recipient alloantigens to donor T cells. It is established in the latter stages of the alloresponse when direct allorecognition diminishes (Baker et al., 2001). However, indirect allorecognition is now also being considered as an inducer of GvHD (Matte et al., 2004).

DCs have been implicated in the development of GvHD following BMT (Clark and Chakraverty, 2002). Several reports have shown a role for host DCs in GvHD (Shlomchik et al., 1999; Ruggeri et al., 2002). The depletion of host DCs has reduced the incidence of the GvH effect (Klangsinsirikul et al., 2002). Donor DCs have only recently been shown to be involved in the development of GvHD (Matte et al., 2004). The target tissue is also important in determining the involvement of host or donor APCs. It has been shown that host APCs are dominant in skin disease, whereas donor APCs were required for gut GvHD providing a reason to target either donor or host APCs rather than host APCs solely (Anderson et al., 2005). As both donor and recipient DCs play an important role in direct and indirect allorecognition, DCs may be relevant in the GvH effect.

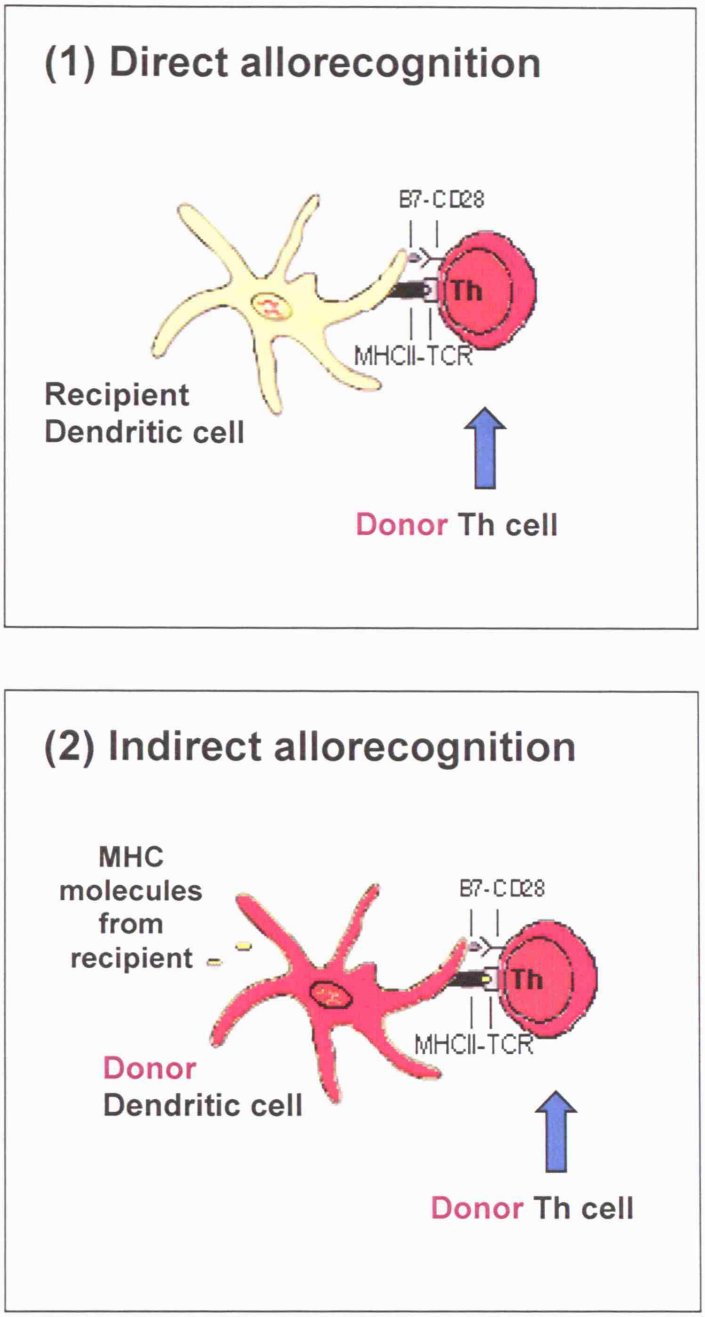


Figure 1.11. The proposed mechanisms for the development of graft versus host disease.

The two pathways of T cell activation during alloreactivity:

1. Direct allorecognition: donor T cells bind directly to recipient MHC-peptide complexes on recipient DCs.

2. Indirect allorecognition: donor T cells recognise recipient MHC-peptide complexes presented by donor DCs.

1.10 Hypothesis

The reduced incidence and severity of GvHD following CBT when compared to BMT is influenced by the immunological characteristics of DC subsets present in the CB.

1.10.1 Aim of the study

To test this hypothesis, phenotypic and functional analyses of DC subsets isolated from CB were performed and compared to those from APB. To determine various characteristics, the following experiments were undertaken on MDCs and PDCs present in CB:

a. The immunophenotyping of MDCs and PDCs.

b. The study of cytokine profiles of stimulated MDCs and PDCs.

c. The assessment and comparison of the endocytic capacity of MDCs and PDCs.

d. The investigation of the allostimulatory capacity of both MDCs and PDCs *in vitro*.

e. The capacity of cytokine secretion following allogeneic stimulation by MDCs and PDCs.

Chapter II

MATERIALS AND METHODS

2.1 Materials

The following materials were necessary to undertake the various investigations:

APB serum (pooled in-house, NBS)

Agarose (Sigma-Aldrich, Dorset, UK)

AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany)

β-mercaptoethanol (Sigma-Aldrich)

Beadlyte® reagents (Upstate, Dundee, UK)

Betaine monohydrate (Fluka, Sigma-Aldrich)

Beta counter (Matrix 96, Perkin-Elmer, Wellesley, MA, USA)

Bovine Serum Albumin (BSA) (Biosera, Ringmer, UK)

Cell isolation kits from Miltenyi Biotec for the following cell types:

a. monocytes
b. T cells
c. Total dendritic cells (DCs)
d. Myeloid DCs (MDCs)
e. Plasmacytoid DCs (PDCs)

Centrifuge (Jouan, St Herblain, France)

Centrifuge 5415 D (Eppendorf, Hamberg, Germany)

CFSE ((5- and 6-) carboxyl fluorescein disuccinyl ester) (Fluka, Sigma-Aldrich)

Chambered coverglass (Lab-Tek, VWR International Ltd, Leicester, UK)

CO_2 incubator (Sanyo electric Biomedical Co Ltd, Japan)

Cryovials (Nalgene, Hereford, UK)

Dimethylsulphoxide (DMSO) (Sigma-Aldrich)

DNase 1 (Qiagen, Crawley, West Sussex, UK)

Deoxynucleotide triphosphates (dNTPs) (Helena Biosciences, Sunderland, UK)

Dulbecco's Phosphate Buffered Saline (PBS) (Sigma-Aldrich)

Ethylenediaminetetraacetic acid (EDTA) (Sigma)

ELISA reader (Labsystems iEMS Reader MF, Cambridge, UK)

Ethidium bromide (Sigma-Aldrich)

Fetal Calf/Bovine Serum (FCS/FBS) (Invitrogen, Paisley, UK)

Ficoll Hypaque (Nycomed, Oslo, Norway)

Fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich)

Fix and permeabilisation kit (Beckman Coulter, High Wycombe, UK)

Flow-count fluorospheres (Beckman Coulter)

Flow cytometer:

a. FACScan for phenotyping (Becton Dickinson, San Jose, CA, USA)
b. FC500 for absolute cell counts (Beckman Coulter)
c. Luminex 100® technology for cytokine detection (Qiagen)

γ-irradiator (CIS BioInternational, France)

21-gauge needle (Becton Dickinson)

Glutamine (GibcoBRL, Life Technologies, Paisley, UK)

Harvester (Filtermate 196, Perkin-Elmer)

Magnetic columns (Miltenyi Biotec)

Penicillin/Streptomycin (GibcoBRL)

PCR thermal cycler (Helena Biosciences)

Primers (Invitrogen)

Red cell lysis solution (Beckman Coulter)

Reverse Trancriptase (RT) kit (Promega, Madison,WI, USA)

Rinsing buffer (PBS + 2mM EDTA)

RNeasy Mini kit (Qiagen)

RPMI (Roswell Park Memorial Institute) medium (Sigma-Aldrich)

Running buffers (PBS + 2mM EDTA + 0.5% BSA)

Spectrophotometer (Biomate3, ThermoSpectronic, Rochester, NY, USA)

Trypan blue solution (Sigma-Aldrich)

Taq polymerase (Helena Biosciences)

^{3}H Thymidine (Amersham, Buckinghamshire, UK)

UV-image analyser (GRI, Essex, UK)

Vacuum manifold (Millipore, Molsheim, France)

Waterbath (Grant, Cambridge, UK)

24- and 96-well culture plates (Falcon, Becton Dickinson)

2.1.1 Monoclonal antibodies.

Murine monoclonal antibodies (MoAbs) and isotype controls of different specificities were used (see Appendix 1). The main MoAbs used to differentiate between the two DC subsets were HLA-DR peridinin chlorophyll protein (PerCP), BDCA1 (CD1c), BDCA2 (CD303), CD11c and CD123 allophycocyanin (APC).

2.1.2 *Blood samples*

Cord blood: CB was obtained from normal full-term deliveries (40 ± 2 weeks) collected following Ethical Committee approval and consent from willing mothers. The collections were performed at the following hospitals: Northwick Park and Barnet Hospitals, London, and The John Radcliffe Hospital, Oxford. The collection of CB was performed as shown in Figure 2.1.

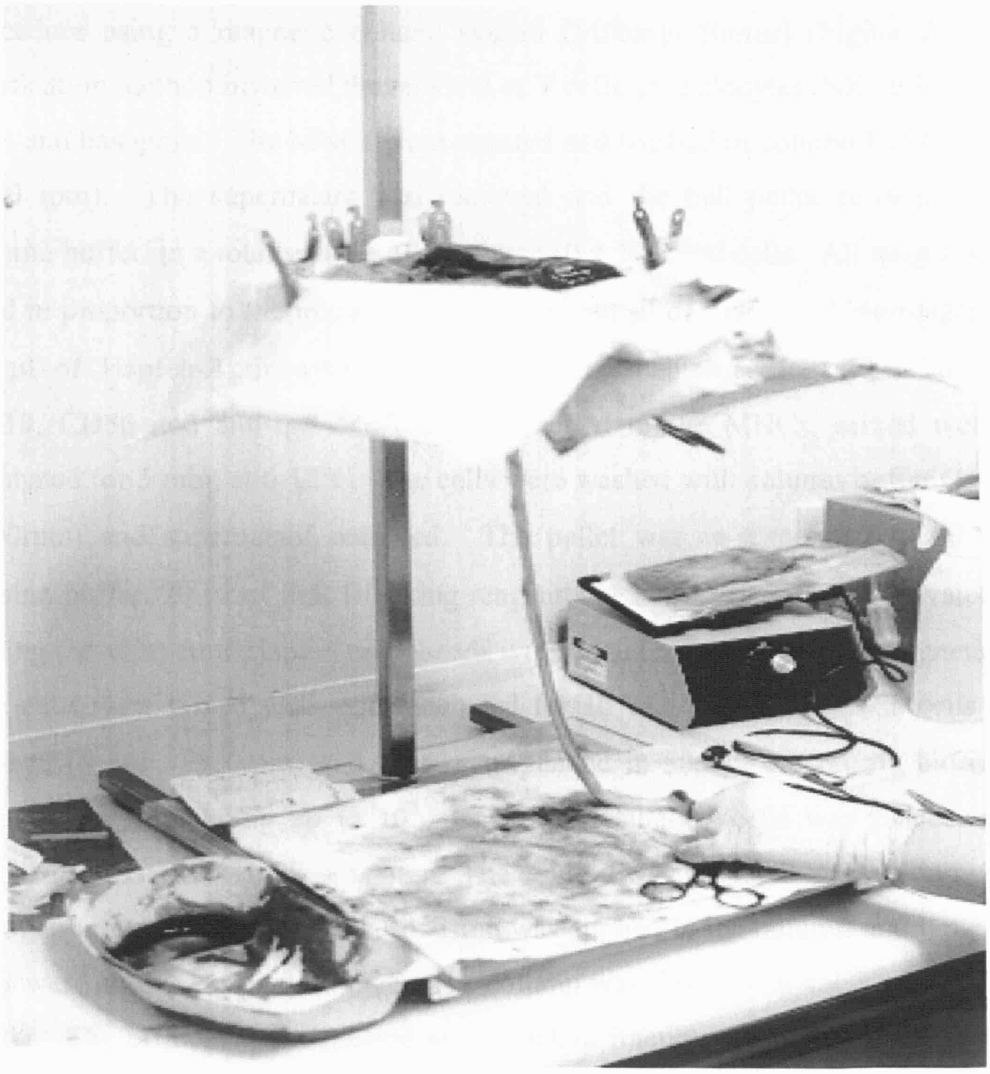
Buffy coats: Buffy coats containing leucocytes were obtained from healthy donors between the ages of 17 and 65 years which were supplied by the NBS (Colindale, North London, UK).

2.2 Methods

2.2.1 *Cell separation techniques*

2.2.1.1 *Mononuclear cells*

Fresh human APB mononuclear cells (MNCs) and CB MNCs were obtained from buffy coats or whole blood, using the Ficoll density gradient centrifugation method. Buffy coats were diluted 1 in 6 and the CB was diluted 1 in 3, with PBS. The diluted blood was then layered over 15 ml of Ficoll per tube. Tubes were centrifuged for 25 min at 2500 rpm to obtain the MNC layer, which was then washed twice with PBS (5 min, 1600 rpm). MNCs were used immediately for cell subset separation or otherwise frozen using the DMSO method. The freezing process involved aliquoting up to a maximum of 50 x 10^6 MNCs in 0.5 ml of FCS solution (80% FCS, 20% RPMI) and then 0.5 ml of DMSO solution (80% RPMI, 20% DMSO). 1 ml of cells was placed in cryovials for overnight storage in a freezing container at -70^0C, and then placed into liquid nitrogen at -180^oC, until required. Before cell subset separation, if frozen, MNCs were rapidly thawed in a waterbath at 37°C. 1 ml of cells (up to 50 x 10^6 MNCs) were added to 20 ml of RPMI medium and washed (5 min, 1600 rpm). Cell counts and viability were assessed using trypan blue solution. If viability was below 90%, dead cells were removed, in which a 10 ml cell suspension was layered over 5 ml of Ficoll, centrifuged for 25 min and the cells were washed twice with PBS.



Photograph supplied by the cord blood bank

Figure 2.1. The cord blood collection process.

This image depicts the method of cord blood collection following delivery of the placenta. The placenta was initially suspended using clamps. The cord was thoroughly disinfected before the vein was cannulated. The blood then drained via gravity into a bag containing anti-coagulant with constant agitation. Once collected, the bag was sealed and stored at room temperature. The samples were then processed within 24 hours of collection.

2.2.1.2 Monocyte isolation

Monocytes were isolated from the MNC suspension by a negative selection procedure using a magnetic column system (Miltenyi Biotec) (Figure 2.2). This purification method involved the removal of T cells, granulocytes, NK cells, B cells, DCs and basophils. The MNCs were counted and washed in column buffer (5 min, 1600 rpm). The supernatant was removed and the cell pellet re-suspended in column buffer, in a total volume of 60 µl per 10 x 10^6 total cells. All reagents were used in proportion to the initial MNC number. 20 µl of FcR Blocking reagent and 20 µl of Hapten-Antibody Cocktail (containing hapten-conjugated CD3, CD7, CD19, CD56 and anti-IgE MoAbs) were added to the MNCs, mixed well and incubated for 5 min, at 6-12°C. The cells were washed with column buffer (10 min, 1600rpm) and supernatant removed. The pellet was re-suspended in 60 µl of column buffer. 20 µl of FcR Blocking reagent and 20 µl of magnetic activated cell sorting (MACS) Anti-Hapten microbeads were added to label the cells magnetically. The cells were mixed well, and incubated for 15 min at 6-12°C. The cells were washed (5 min, 1600rpm) and then re-suspended in 500 µl of column buffer. A column accommodating up to 10^8 magnetically labelled cells was placed in the magnetic field of a MACS separator. The column was prepared by washing with 3 ml of column buffer. The cell suspension was applied to the column and unlabelled cells were allowed to pass through. The column was rinsed with 4 x 3 ml of column buffer. The effluent was collected as a negative fraction representing the enriched monocyte fraction. Purified monocytes were >98% $CD14^+$ assessed by flow cytometry.

A bench-top automated magnetic cell sorting system, the autoMACS, eventually replaced manual separation of monocytes. There was a relevant program for each separation involving both positive and negative selections. For monocytes a negative selection was appropriate and the 'DEPLETE' program was used with the cells being released from the 'neg1' port.

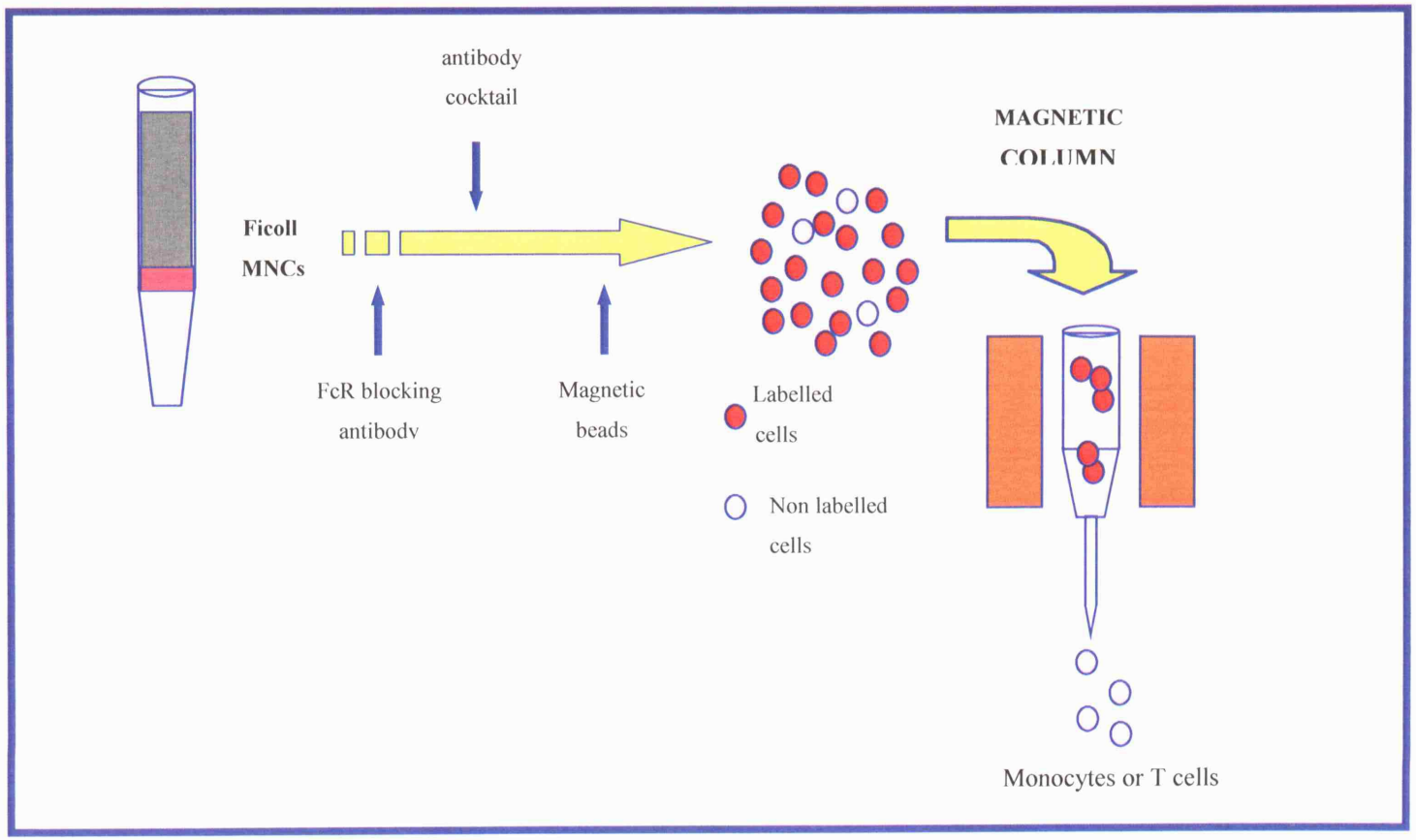


Figure 2.2. Separation of monocytes or T cells from adult peripheral blood.

Monocytes and T cells were isolated using a cocktail of specific MoAbs and magnetic beads to remove unwanted cell types. The separation involved a negative selection releasing unlabelled cells.

2.2.1.2.1 Generation of monocyte derived dendritic cells (MDDCs)

After obtaining purified (98% pure) $CD14^+$ monocytes determined by flow cytometry, MDDCs were generated by culturing the monocytes at a density of 5 x 10^5 monocytes per ml. The cells were cultured for 7 days in complete medium (RPMI containing 10% FCS, 250 μg/ml of penicillin/streptomycin, 2 mM L-glutamine and 25 mM HEPES (GibcoBRL)), plus GM-CSF (70 ng/ml) and IL-4 (35 ng/ml) (Peprotech, NJ, USA) at 37°C in a 24-well flat-bottomed plate. The medium was changed every 2 days using complete medium and the same cocktail of cytokines. On day 7, non-adherent cells were removed from the 24-well plate and considered as immature MDDCs. The phenotypic characteristics and purity of the cells were evaluated by flow cytometric analysis. This was to assess the generation of MDDCs following culture. As they are myeloid derived, MDDCs were HLA-DR^+ and $CD11c^+$ DCs. The MDDCs were used as a positive control for endocytosis studies.

2.2.1.3 Purification of circulating dendritic cells

The isolation of circulating DCs involved a two step purification method of negative and positive selection using the MACS 'total blood DC isolation' kit (Miltenyi Biotec) (Figure 2.4). This isolation method involved the deletion of T cells, NK cells, and monocytes by indirect labelling of cells using a cocktail of MoAbs (CD3, CD16, and CD11b). MNCs obtained from buffy coats were re-suspended in 300 μl of column buffer for every 100 x 10^6 cells. Anti-FcR blocking antibody was added at a concentration of 1 x 10^9 cells/ml of column buffer, along with the same concentration of the Hapten antibody cocktail. The cell suspension was mixed prior to incubation at 6-12°C for 10 min. The cells were washed with column buffer at 1600 rpm for 5 min. The supernatant was removed and the pellet re-suspended in 900 μl of column buffer for every 100 x 10^6 cells. To this, anti-hapten microbeads were added and cells incubated at 6-12°C for 15 min. A pre-cooled column was pre-wet and washed with 3 ml of column buffer. A 1 ml cell suspension was then added to the column followed by 3 x 3 ml of column buffer. The negative (or DC pre-enrichment) fraction passed through the column untouched and was washed by centrifugation at 1600 rpm for 5 min. Alternatively, the autoMACS was used selecting the 'DEPLETES' program. This selection is for rare cells and to achieve the isolation of a highly pure cell population. The cells were eluted from the 'neg1'

port. This was followed by a positive selection based on the direct labelling of cells with CD4 microbeads. The pellet was re-suspended in column buffer at a concentration of 1 x 10^9 cells/ml. For every 100 x 10^6 cells, 100 µl of CD4 microbeads were then added and the cell suspension incubated at 6-12°C for 30 min, centrifuged at 1600rpm for 5 min and re-suspended in 500 µl for every 100 x 10^6 cells. A pre-cooled 'MS' column was attached to the adapter magnet and washed with 500 µl of column buffer. The cell suspension was then passed through and further washed with 3 x 500 µl of column buffer. The negative fraction (flow-through) was discarded and the column containing the positive fraction was removed from the magnet and washed with 1 ml of column buffer, using a plunger (for flow diagram refer to Appendix 1). With the autoMACS, 'POSSELD' was the selected program and the cells were dispensed at the 'pos2' port. Once collected, a cell count was performed on the DCs. DCs were assessed for their purity and phenotype by flow cytometric analysis using specific MoAbs. For MDCs, CD11c and HLA-DR, and for PDCs, CD123 and HLA-DR, were the markers of identity.

2.2.1.4 Purification of the dendritic cell subsets

DC subsets can be separated by integrating novel DC specific markers called Blood Dendritic Cell Antigens (BDCAs) (Miltenyi Biotec). This technique is based on the differential and specific expression of BDCA in the distinct DC subsets. With this methodology, both the myeloid (BDCA1 or $CD1c^+$) and the plasmacytoid (BDCA4 or $CD304^+$) DCs could be separated and used to study their phenotypic and functional characteristics. Both MDCs and PDCs were isolated from the same sample for all experiments for comparable results.

2.2.1.4.1 Myeloid dendritic cell subset

The isolation of the MDC subset involved a two step purification process, using the MACS 'CD1c (BDCA1) DC isolation kit' (Miltenyi Biotec). As the CD1c antigen is expressed on both MDCs and B cells, MNCs were initially depleted of B cells using CD19 microbeads. MNCs were re-suspended in 200 µl of column buffer for every 100 x 10^6 cells. 100 µl each of FcR blocking reagent, CD19 microbeads and

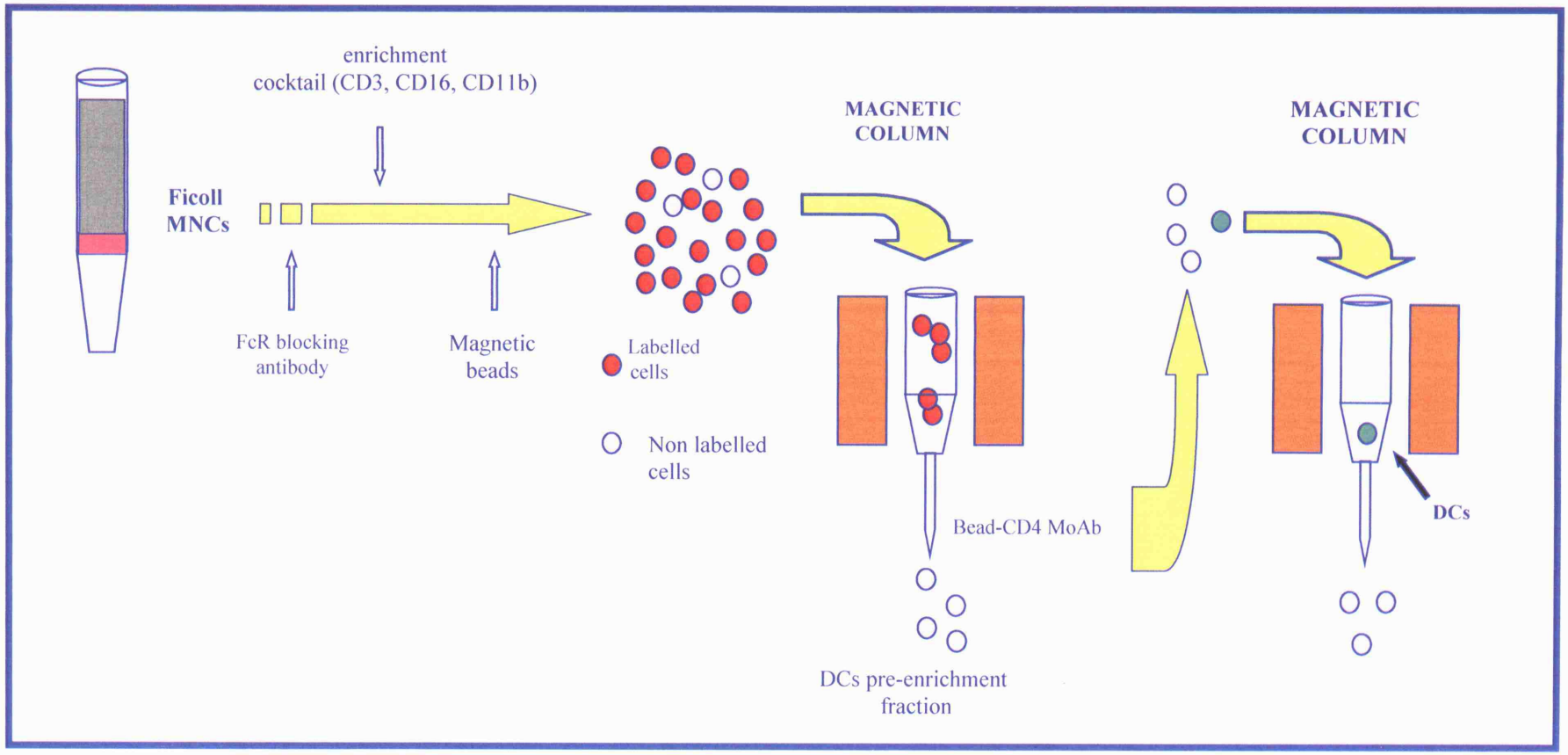


Figure 2.3. Purification of circulating dendritic cells.

Dendritic cells were isolated using a magnetic cell sorting system (MACS), which involved a negative selection to remove the T, NK cells and monocytes. The separation process was continued with the DC pre-enrichment fraction. A positive selection involved obtaining the dendritic cells labelled with anti-CD4.

CD1c-biotin antibody were added to the cell suspension. The cells were thoroughly mixed and incubated for 15 min at 6-12°C. The cells were washed with column buffer (5 min, 1600 rpm), supernatant completely removed and re-suspended in a final volume of 500 µl of column buffer per 100 x 10^6 total cells. Depending on the cell number, the relevant MACS column was chosen. A column used for 100 x 10^6 $CD19^+$ cells was primed by washing it with 2 ml of column buffer ready for separation. The cell suspension was then applied to the column, with the negative cells passing through as the B cell depleted fraction. The column was rinsed twice with 1 ml of column buffer, until the entire flow-through had been collected. The manual separation system was replaced with the autoMACS. The 'DEPLETES' program was used to deplete B cells, and as this is the sensitive mode it generated greater purity. The cells were eluted at the 'neg1' port.

The next step involved a positive selection of $CD1c^+$ blood DCs. After centrifuging the B cell depleted fraction, the cell pellet was re-suspended in 400 µl of column buffer for every 100 x 10^6 total cells. Anti-biotin microbeads (100 µl) were added, the cells were mixed well and incubated for 15 min at 6-12°C. The cells were then washed with column buffer, centrifuged and re-suspended in a final volume of 500 µl of column buffer per 100 x 10^6 total cells. Again, depending on the number of cells available, a MACS column was selected. The column was pre-wet, and the sample was added. Column buffer was used to wash the column three times. The column was then removed from the magnet and placed on a new collection tube. 1 ml of column buffer was added to the column and using a plunger the positive cells were directly flushed out (for flow diagram refer to Appendix 1). These cells were then counted and the purity was evaluated. Using the autoMACS, the selection of the 'POSSELD' program eluted the cells at 'pos2' port. Cell purity as assessed by flow cytometry, was more than 80% manually but increased to 95% with the introduction of the automated system (see Chapter 3, Figure 3.2). The 'POSSELD2' program, which loaded the samples onto the column faster and dispensed a larger final volume was used to elute CB MDCs.

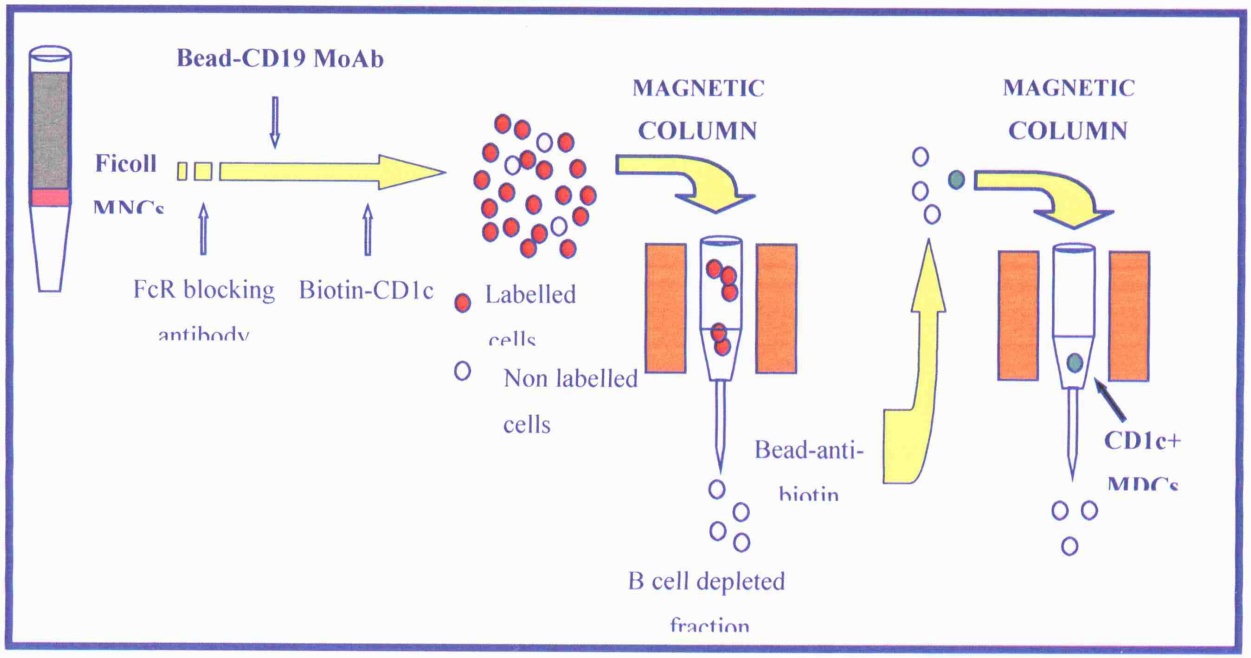


Figure 2.4. Purification of the myeloid dendritic cell subset.

Myeloid DCs were isolated using the magnetic cell sorting system (MACS). Specific antibodies were used to select this subpopulation. B cells were initially removed using the CD19 MoAb, with only the B cell depleted fraction passing through the magnetic column. The second incubation with anti-biotin beads was followed by a positive selection for the $CD1c^{+}$ MDCs.

2.2.1.4.2 Plasmacytoid dendritic cell subset

A direct magnetic labelling system based on anti-CD304 microbeads was used to isolate PDCs (Figure 2.5). The method for PDC isolation was similar but simpler than the isolation of MDCs as it involved only a positive selection. MNCs were re-suspended in 300 µl of column buffer per 100 x 10^6 total cells. 100 µl of FcR blocking reagent and 100 µl of anti-CD304 microbeads were mixed well with the cells before incubation for 15 min at 6-12°C. The cells were washed with column buffer, centrifuged, and the pellet re-suspended in a final volume of 500 µl per 100 x 10^6 cells. An 'MS' column was used for the separation, which was pre-wetted with 3 ml of column buffer before addition of the cell suspension. The column was then washed with 3 x 3 ml of column buffer. The column was removed from the magnet and placed on a new tube. 3 ml of column buffer was added to the column and using a plunger the positive cells were directly flushed out (for flow diagram refer to Appendix 1). These cells were then counted and purity was assessed. Magnetic separation using the autoMACS involved selecting the 'POSSELD' program, and the cells of interest were collected from the 'pos2' port. The introduction of the autoMACS dramatically increased the cell purity from 60% to 95% (see Chapter 3, Figure 3.1). The 'POSSELD2' program was selected to isolate PDCs present in CB.

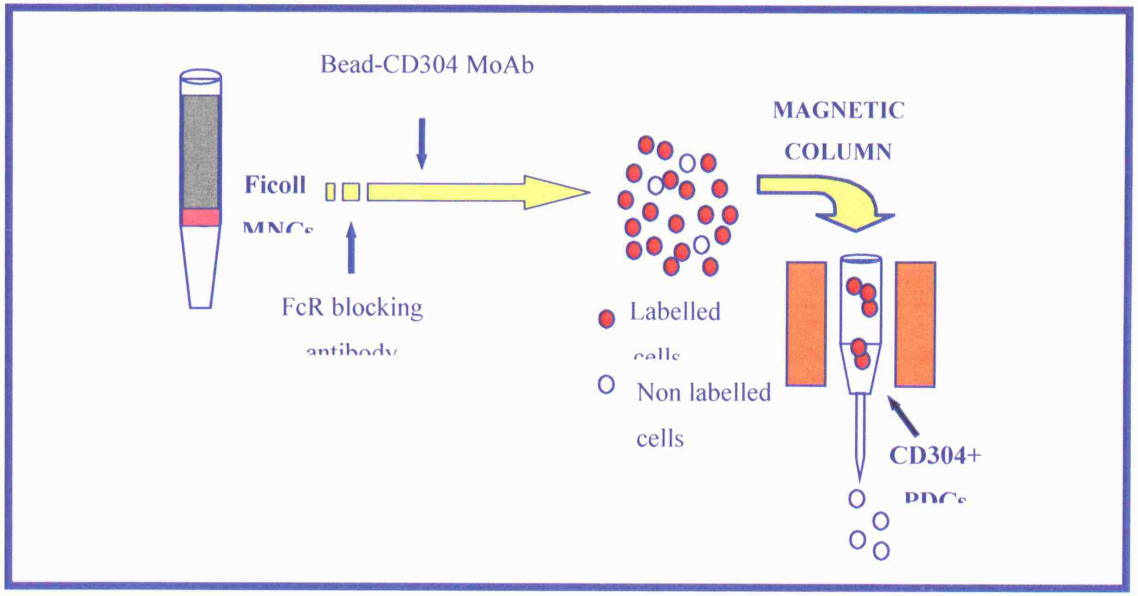


Figure 2.5. Purification of the plasmacytoid dendritic cell subset.

Plasmacytoid DCs were isolated using the magnetic cell sorting system (MACS) from Miltenyi Biotec. A specific antibody, CD304, was used to select this cell subpopulation, involving only a positive selection.

2.2.1.5 Separation of T cells

T cells were isolated using the MACS 'Pan T cell isolation kit' (Miltenyi Biotec) (Figure 2.2). Cells were counted and re-suspended in 160 µl of column buffer for every 20 x 10^6 cells. 40 µl of Hapten Antibody Cocktail (containing MoAbs for CD14, CD16, CD19,) was added, cells mixed and incubated for 10 min at 6-12°C. Following two washes, the pellet was re-suspended in 160 µl of column buffer. 40 µl of MACs Anti-Hapten microbeads were added, the cells mixed and incubated for 15 min at 6-12°C. This was followed by a further wash step. An 'LS' column was prepared by rinsing it with 3 ml of column buffer. The pellet was re-suspended in 1 ml of column buffer, and cell suspension added to the column. The column was rinsed with 4 x 3 ml of column buffer. The negative fraction containing T cells was collected and cells were counted (refer to Figure 2.2). With the introduction of the autoMACS the 'DEPLETE' program was used and the purity of the cells assessed by flow cytometry was 98%. The isolated T cells were used as the negative control for endocytosis studies and as the responder cell population in Mixed Lymphocyte Reaction (MLR) assays (see later).

2.2.2 Flow cytometry

2.2.2.1 Absolute cell counting

The determination of absolute cell counts is an effective method of establishing frequencies of cells in blood samples. A reagent incorporating fluorospheres (Beckman Coulter) is used to directly determine absolute counts of the various lymphocyte subsets in whole blood using flow cytometry. The lymphocyte population of APB is composed of three cell types. These include the morphologically similar T, B and NK cells, which can be identified by characteristic antigenic differences in their cell membranes. Various MoAbs define specific, discrete T, B and NK cell surface antigens and are used to identify and enumerate percentages and absolute counts of these lymphocyte populations. The standard method for obtaining absolute cell counts (cells/µl) is known as a dual-platform technique, which combines the counts from a haematology analyser and flow cytometer. The formula required is:

Absolute count (cells/μl) = total white blood cell count (cells/μl) x % lymphocytes (from haematology analyser) x % positively stained cells / 10^4 (from a flow cytometer)

The accuracy of this method is reliant on two different instruments resulting in greater error. Therefore, the use of only one instrument, i.e. the flow cytometer, should improve the accuracy, and is referred to as the single platform technique. This new method employs the use of 'Flow-Count' fluorospheres (Beckman Coulter) in the sample tested directly obtaining results from the flow cytometer. The polystyrene fluorospheres are 10 μm in diameter and present in aqueous suspension medium containing a surfactant and 1 % formaldehyde.

The technique is based on the premise that the volume of cells added to the sample tube is exactly the same volume as the fluorosphere suspension (ratio of cell suspension to bead suspension is 1:1). The fluorosphere concentration is pre-determined (provided by the manufacturer) and is used as a reference to compare the number of cells in the sample. So, the absolute cell count may be calculated from the following equation:

Absolute cell count (cells/μl) =

$$\frac{\text{Total cell number counted}}{\text{Total number of fluorospheres}} \times \text{'Flow-Count' fluorospheres assayed concentration}$$

The 'Flow-Count' method was reliant on mixing a known volume (100 μl) and concentration of fluorospheres with the same volume of whole blood. The method involved labelling the whole blood sample with the relevant MoAb for 15 min at room temperature (RT). The red blood cells were then lysed by the addition of 500 μl of Optilyse C (Beckman Coulter) and briefly vortexed. The cells were incubated at RT for 10 min, up to a maximum of 2 hr after which time 500 μl of PBS was then added and vortexed. The 'Flow-Count' fluorospheres were initially mixed for 10-12 sec (avoiding excessive mixing to minimise air bubbles) and 100 μl added to the

tube, vortexed for 5 sec. The cells were also vortexed before flow cytometric analysis, which was performed within 2 hr of fluorosphere addition.

2.2.2.2 Immunophenotyping

Phenotypic analysis was performed on freshly isolated DC subsets using flow cytometry following labelling of cells with directly conjugated MoAbs. For membrane labelling, cells were suspended in PBS containing 0.5% BSA, incubated with the relevant MoAbs for 15 min at RT and washed twice (5 min, 1600rpm) with PBS. For intracellular labelling the cells were then fixed and permeabilised according to the manufacturer's protocol (Beckman Coulter). Initially, the cells were incubated with fixing reagent (Reagent 1; Beckman Coulter) for 15 min at RT, in the dark. The cells were washed and incubated with permeabilising reagent (Reagent 1; Beckman Coulter) for 5 min at RT, in the dark without mixing. The cells were then mixed for 2-3 sec and then various MoAbs were added for 15 min at RT, in the dark.

Following incubation, for both membrane and intracellular labelling, the cells were finally washed, re-suspended in PBS containing 2% FCS and 1% paraformaldehyde (PFA), and analysed by flow cytometry (Becton Dickinson) using the standard CellQuest^{TM} acquisition software (Becton Dickinson). All experiments included isotype controls and were gated using forward and side scatter to exclude dead cells defined by their lower forward scatter and higher side scatter than living cells. At least 5,000 gated events were analysed using the WinMDi software. The results were expressed as percentage of positive cells and the level of expression in terms of Mean Fluorescence Intensity (MFI) of a particular marker was also obtained.

2.2.3 Confocal Microscopy

Confocal microscopy was used to view the distribution of molecules involved in antigen processing and the distribution of FITC-dextran during endocytosis of MDCs and PDCs. The two DC subsets were isolated using MACS technology and labelled, before examination under the microscope. To study the distribution of surface HLA-DR and intracellular HLA-DM, following DC subset isolation, 2 x 10^4 were cultured with their appropriate cytokines, IL-4 for MDCs and IL-3 for PDCs,

overnight at 37°C in a chambered coverglass for immunocytochemistry. Following culture, the cells were labelled with FITC-conjugated anti-HLA-DR for surface HLA-DR expression followed by PE-conjugated anti-HLA-DM, to study intracellular HLA-DM (refer to section 2.2.2.2 for incubation times). For endocytosis studies, the cells were labelled with FITC-dextran and incubated for 6 hr (refer to section 2.2.5.1 for method). The cells were placed on a slide for the detection of dextran uptake. Confocal images were obtained at a magnification of x100. Confocal microscopy is a three-dimensional observation of fluorescence distribution in fixed or live cells. The images were captured in Professor Fernandez's laboratory (University of Essex, Colchester, UK).

2.2.4 Cytokine secretion

The cytokine profile of DC subsets in the steady state and following their stimulation was defined using a multiple cytokine detection system, applying the Luminex® technology. Figure 2.6 describes the principles of the technique. This technique was also used to detect the secretion of cytokines following allogeneic stimulation of DCs with T cells (see section 2.6.2). Additionally, the ELISA method of cytokine detection was used for the detection of IFNγ and IL-4 secretion following allogeneic stimulation of DCs with T cells (Figure 2.7).

2.2.4.1 Stimulation experiments

Once isolated, the two DC subsets were treated with the appropriate stimuli to induce cytokine secretion. IL-4 (50 ng/ml) and IL-3 (50 ng/ml) (Peprotech) were used to examine steady state cytokine secretion by MDCs and PDCs, respectively. LPS (100 ng/ml) (Sigma-Aldrich) and CpG ODN (5 μg/ml) (Invitrogen), were used to specifically stimulate MDCs and PDCs, respectively. The cells were cultured and supernatants removed at time points of 12, 18 and 24 hr, to determine the kinetics of cytokine release. The supernatants were removed and stored at -70°C until ready for cytokine profiling. The supernatants were also removed following allogeneic stimulation of DCs with T cells (MLR assays).

2.2.4.2 Multiple cytokine detection

The technique to study cytokine secretion from stimulated DC subsets and following allogeneic stimulation of DCs with T cells, was a bead-based immunoassay incorporating the Luminex® technology. The major advantage of this method over other cytokine detection systems is its multiplexing capability. Up to 100 potential cytokines can be studied due to the presence of 100 fluorescently labelled bead sets, each specific for a particular cytokine (Figure 2.6).

Initially, the 5000 pg standard, consisting of the 13 cytokines was reconstituted in 2 ml of complete medium and vortexed for 15 sec. A final concentration of 2500 pg/ml was then placed on ice for 5 min. A sample serial dilution of the following concentrations was then performed: 1000, 250, 125, 62.5, 31.2, 15.6 and 0 pg/ml. The filter plate was pre-wet with 25 µl of assay buffer (PBS at pH 7.4, 1% BSA, 0.005% Tween 20, 0.05% sodium azide) per well, vortexed and a vacuum applied to the bottom of the plate. The bottom of the plate was blotted on a paper towel to remove excess buffer. When tissue culture medium was used, 50 µl of standard or sample was added to each well in duplicate. The standards were added in increasing concentration and adjacent to each other. The Beadlyte® anti-human multi-cytokine beads were vortexed at high speed for 30 sec. 25 µl of the bead solution was then added to each well. The plate was covered, mixed by vortexing at low speed and incubated for 2 hr in the dark at RT on a plate shaker. Following incubation, a vacuum manifold was applied to the bottom of the filter plate to remove liquid but avoiding over-drying. The bottom of the plate was blotted on a paper towel. 50 µl of assay buffer was dispensed into each well, the plate vortexed and vacuum repeated followed by the addition of 75 µl of assay buffer to all wells. The plate was covered and mixed by vortexing at low speed. 25 µl of Beadlyte® anti-human multi-cytokine biotin reporter was added to each well. The plate was then covered and mixed by vortex at low speed. The plate was incubated for 1.5 hr in the dark at RT on a plate shaker. Following this, the Beadlyte® Streptavidin-Phycoerythrin was diluted 1:25 in assay buffer and 25 µl of this diluted solution was added to each well. The plate was covered, mixed as before and incubated for 30 min in the dark at RT on a plate shaker. 25 µl of stop solution was then added, the plate vortexed gently, and left for 5 min in the dark at RT with constant agitation. The vacuum

manifold was applied to the bottom of the filter plate to remove the liquid, and the plate blotted dry. Finally, 125 µl of assay buffer was added to the wells and the plate was vortexed at low speed. The plate was then read on the Luminex 100® instrument and results analysed using the Luminex® analysis software.

2.2.4.3 Enzyme Linked Immunosorbent Assay (ELISA)

The cytokines investigated using the ELISA method were the Th1 and Th2 cytokines IFNγ and IL-4, respectively. The ELISA method is referred to as a capture or sandwich ELISA (Figure 2.7). This enables the cytokine to bind with high affinity, concentrating it on the surface of the plate, even with very low concentrations of cytokine. A secondary biotinylated antibody recognises a different epitope to the immobilised primary antibody and was used to detect any bound cytokine.

Initially, the wells of a 96-well plate were coated with 100 µl of capture antibody (BD Biosciences) (a dilution factor of 1:250 for both IL-4 and IFNγ) diluted in coating buffer (0.1 M sodium carbonate, pH 9.5; BD Biosciences). The plate was sealed and incubated overnight at 4°C. After an overnight incubation, the wells were aspirated and the plate washed 3 times with ≥300 µl/well of wash buffer (PBS + 0.05% Tween-20). After the last wash, the plate was inverted and blotted on absorbent paper to remove any residual buffer. The plate was then blocked with ≥200 µl/well of assay diluent (PBS and 10% FBS) and incubated at RT for 1 hr. Following incubation, the wells were aspirated and washed three times as before. The standards and sample dilutions were prepared in assay diluent. 100 µl of each standard and sample was pipetted into their appropriate wells. The plate was sealed and incubated for 2 hr at RT. The wells were then aspirated and washed 5 times with assay buffer. 100 µl of working detector solution (biotinylated detection antibody + avidin-Horse Radish Peroxidase) was added to each well. The plate was sealed and incubated for 1 hr at RT. The wells were then aspirated and washed 7 times, soaking the wells in wash buffer for 30 sec to 1 min for each wash. 100 µl of substrate solution (0.03% Tetramethylbenzidine and 0.05% Hydrogen Peroxide) was added to each well. The plate was incubated for 30 min at RT in the dark. To terminate the reaction, 50 µl of stop solution (1 M phosphoric acid) was added to

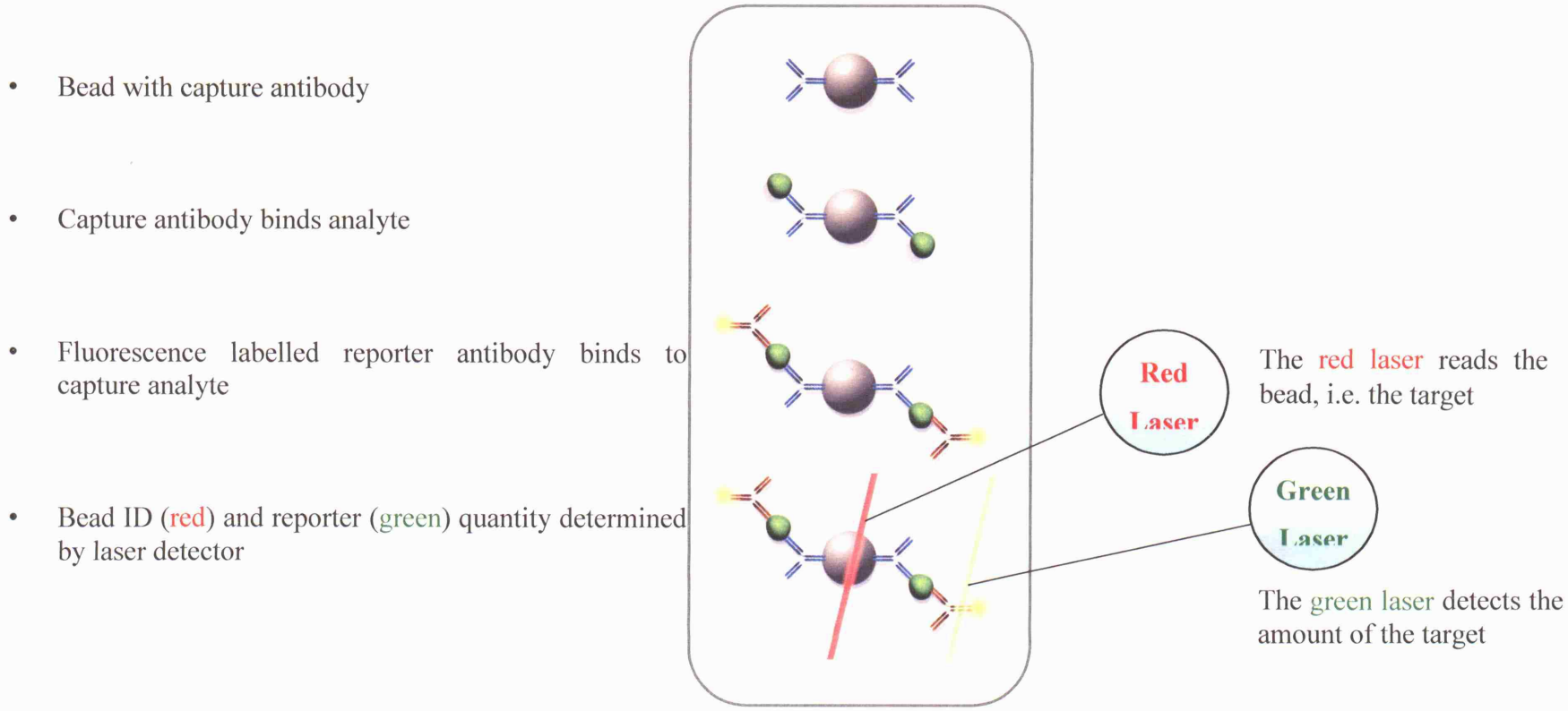


Figure 2.6. Luminex technology for multiple cytokine detection.

The Luminex technology is based on an ELISA (bead-based sandwich immunoassay) and is adapted from the 'Upstate' figure.

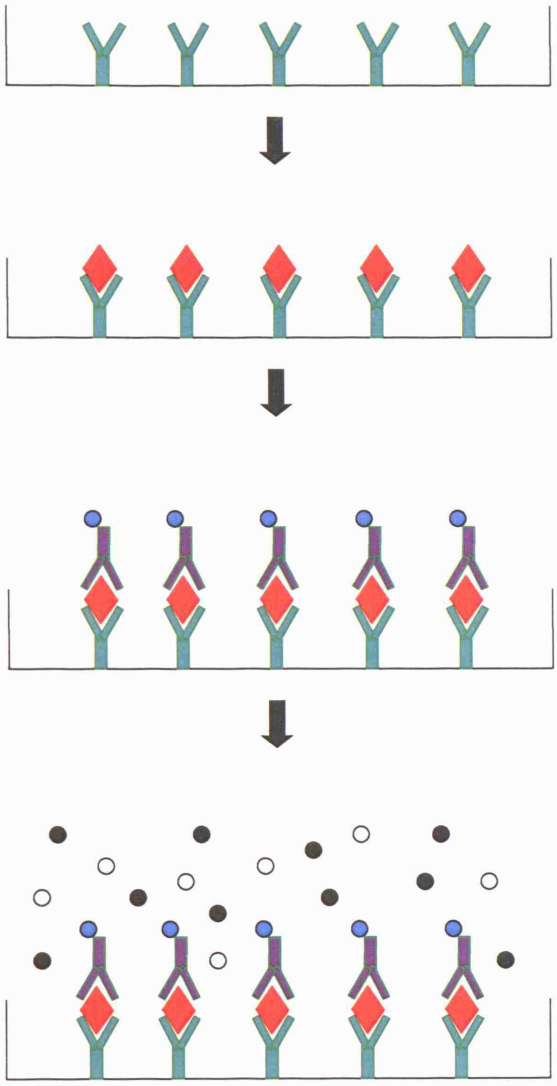


Figure 2.7. The principles of an Enzyme Linked Immunosorbent Assay (ELISA).

(A) The specific antibody is bound to the wells of a flat-bottomed plate. (B) The cultured supernatant is then added which contains the antigen of interest (IL-4 or IFNα). (C) To the bound antigen, the enzyme conjugate (detection antibody) is added. (D) This is followed by the substrate solution, which changes colour (• to o) if the enzyme conjugate is bound to the immune complex (anti-cytokine antibody + cytokine). Finally, the stop solution is added to terminate the reaction. The plate is read on an ELISA plate reader.

each well. The absorbance was read on an ELISA reader at 450 nm within 30 min of stopping the reaction. A standard curve was then produced using the Optical Density (OD) results from the various standard concentrations. IL-4 included the following range of concentrations: 7.8, 15.6, 31.3, 62.5, 125 and 250 pg/ml and IFNγ included the standards of 4.7, 9.4, 18.8, 37.5, 75 and 150 pg/ml. The results were then extrapolated from each standard curve.

2.2.5 Functional studies

Following the isolation of specific DC subsets, functional studies were performed initially examining endocytosis by FITC-dextran uptake, and secondly, investigating the allostimulatory capacities of DC subsets using the MLR assay.

2.2.5.1 Measurement of endocytosis using FITC-dextran uptake

Approximately 2 x 10^5 cells per sample were incubated at 37°C for 0, 2, 4 and 6 hr, in media containing 0.5 mg/ml FITC-dextran, which was at a molecular weight of 40,000. After incubation, cells were washed extensively (4 times) with cold PBS, to remove excess dextran and then fixed in cold 1% PFA solution. Background control cells were incubated with cold FITC-dextran, to observe any non-specific dextran binding to the cell surface. The quantitative uptake of FITC-dextran by the cells was determined by flow cytometry, acquiring 10,000 cells per sample. The results were analysed using the WinMDi software. Endocytic activity was expressed as MFI, calculated as MFI of positive cells at 37°C – MFI of positive cells at 4°C. MDDCs were used as the positive control and T cells as the negative control.

2.2.5.2 Mixed Lymphocyte Reaction (MLR) assay

Freshly isolated MDCs and PDCs were the stimulator cells used following irradiation (25-30 Gy). Allogeneic APB T cells were the responder population and following isolation were added to each well at a concentration of 1 x 10^7/ml. Both stimulators and responders were HLA typed to determine mismatches. The DC subsets were seeded with responders at varying DC-T cell ratios (1:10, 1:20 and 1:50) in 96-well round-bottomed plates, in complete medium of RPMI, 10% APB serum, 2 mM L-glutamine and 250 μg/ml of penicillin/streptomycin. The cells were

co-cultured for 5 days at 37°C in 5% CO_2. Each test was set up in triplicate wells. T cell proliferation was initially detected by the formation of clusters within each well observed by light microscopy.

2.2.5.2.1 Measuring T cell proliferation

T cell proliferation was measured using the radioactive technique of ^3H Thymidine incorporation by proliferating cells. 37 kBq/well ^3H Thymidine was added on day 4, 18-20 hr prior to harvesting, which was performed on day 5 of co-culture. The cells were harvested onto self-aligning glass-fibre filters using a semi-automated harvester, and ^3H Thymidine incorporation was detected using a beta counter. Responses obtained were referred to as mean counts per minute (cpm) of triplicate wells.

2.2.6 HLA typing for MLR assays

The extraction of DNA and subsequent HLA typing of samples was performed to obtain HLA disparate DCs and allogeneic T cells for MLR assays.

2.2.6.1 DNA extraction

Following Ficoll separation of buffy coats, the MNC layer was used for the isolation of the various cell populations. The remaining red blood cell pellet was retained and DNA extraction performed using the 'salting out' method. The method involved taking 3 ml of red cell pellet and transferring it into a 15 ml tube. Red cell lysis buffer (w/v 8.28 mg/ml ammonium chloride, 1 mg/ml potassium bicarbonate, 0.37 mg/ml sodium EDTA) was added to remove the red cells by creating a difference in the osmotic potential causing lysis. The tube was allowed to stand for 15 min with occasional inversion. The tube was then centrifuged at 3500 rpm for 10 min resulting in a white cell pellet. The supernatant was removed and if no white cell pellet obtained, i.e. pellet still red, the lysis and centrifugation steps were repeated. The tube was vortexed to re-suspend the pellet. 3 ml of cell lysis solution (w/v 9.3 mg/ml sodium EDTA, 2% sodium dodecyl sulphate (SDS)) were added and the tube shaken vigorously for 15 sec to ensure the cells were lysed. The detergent, SDS, disrupts the white blood cell membrane enabling release of the nucleus. 1 ml of

protein precipitate solution (10 M Ammonium Acetate) was added and the tube was again vigorously vortexed. The tube was centrifuged at 3500 rpm for 20 min and the supernatant removed from the pellet and placed in a fresh tube. 3 ml of 100% isopropanol was added and the tube inverted several times until the DNA fully precipitated. 1 ml of 80% ethanol was pipetted into a 1.5 ml eppendorf tube. To this, the precipitated DNA was added using a pastette and the tube was inverted. The tube was then centrifuged for 1 min at 13,000 rpm and the supernatant discarded. The process from the addition of ethanol was repeated and any excess alcohol removed and the tube left to air dry for 5 min. Between 200 µl and 800 µl of distilled water was added to the tube and the sample placed on a rotator for a minimum of 2 H to enable the DNA to dissolve. DNA was initially stored at 4°C for immediate use or frozen indefinitely at -20°C.

2.2.6.2 HLA Typing

HLA typing was performed on the DNA samples to determine their HLA status with a polymerase chain reaction using sequence specific primers (PCR-SSP). The concentration of DNA was measured using a spectrophotometer. The PCR-SSP was carried out according to the method described by Bunce *et al* (Bunce et al., 1995). The PCR products were then resolved on a 2% w/v agarose gel stained with 0.005% v/v ethidium bromide in 1x TBE buffer (Trizma base, Boric acid and 0.2 M disodium EDTA at pH8) for 21 min at 450V. 5 µl of sample was added to 5 µl of loading buffer (0.125% Orange G, glycerol, 1x TBE buffer). 2 µl of a 1Kb DNA ladder (Helena Biosciences) was run as a molecular weight marker. Gel images were read and photographs obtained using an UV-image analyser.

2.2.7 The determination of mRNA expression

The DC subsets were studied for their expression of HLA-G at the mRNA level. Following isolation of the DC subsets, RNA was extracted and cDNA generated. RT-PCR was then undertaken and results obtained by gel electrophoresis.

2.2.7.1 RNA extraction

The RNeasy Mini kit was used to extract total RNA from various human cells, including monocytes, DCs and T cells. Following isolation of the particular cell types, a maximum of 2 x 10^6 cells were used for each RNA extraction. Cells were spun for 5 min at 800 g (Centrifuge 5415 D) to remove the supernatant. The pellet was loosened and 350 µl of lysis solution (buffer RLT) containing 1% v/v β-mercaptoethanol was added to the cells and vortexed. Each sample was homogenised to shear high molecular weight (MW) genomic DNA, remove cellular debris and reduce viscosity of the lysate. This was achieved by passing the lysate 5-10 times through a 20-gauge needle attached to a syringe. In preparation for binding, 350 µl of 70% ethanol was added to the lysate and mixed using a pipette. The lysate (up to 700 µl) was transferred to an RNA binding column and placed in a 2 ml collection tube. It was spun for 15 sec at 10,000 rpm (Centrifuge 5415 D) and the flow-through discarded. To ensure the removal of all contaminating DNA, the sample was treated with DNase 1. This involved the addition of 350 µl of buffer RW1 onto the column and centrifugation for 15 sec at 10,000 rpm. The flow-through was discarded and 80 µl of RNase free, DNase incubation mix (10 µl of DNase stock solution added to 70 µl of buffer RDD and mixed by inverting the tube and not vortexing) pipetted onto the silica-gel membrane and incubated for 15 min at RT. 350 µl of buffer RW1 was pipetted into the column and centrifuged for 15 sec at 10,000 rpm. Both the flow-through and collection tube were discarded. 500 µl of buffer RPE was pipetted into the column, centrifuged (15 sec at 10,000 rpm) and the flow-through discarded. This was repeated but centrifuged for 2 min at 10,000 rpm to dry the binding column. The binding column was transferred to a new collection tube ready for elution. 50 µl of RNase-free water was added to the centre of the binding column and spun for 1 min at 10,000 rpm. The eluate was collected and the purified RNA stored at -70 °C.

Each sample of RNA was analysed using a spectrophotometer to determine the concentration of RNA. The samples were diluted accordingly and absorbance and concentration of RNA content (µg/µl) determined.

2.2.7.2 Reverse Transcription generation of cDNA

The RNA from each cell type was converted to cDNA using reverse transcription. 1 μg of RNA was placed in a microcentrifuge tube and incubated at 70°C for 10 min. The tube was spun briefly and then placed on ice. A 20 μl reaction was prepared by adding 4 μl of 25 mM $MgCl_2$, 2 μl of 10x RT buffer, 2 μl of 10 mM dNTP mixture, 0.5 μl of recombinant RNasin ribonuclease inhibitor, 0.5 μ of AMV reverse transcriptase, 0.5 μg of $Oligo(dT)_{15}$ primer, and finally RNase-free water to the 1 μg of total RNA to make up a final volume of 20 μl. The reaction was incubated for 15 min at 42°C followed by heating to 95°C for 5 min, and finally incubated for 5 min at 4°C. The first strand cDNA synthesis was then complete and samples were stored at -20 °C.

2.2.7.3 Polymerase Chain Reaction (PCR)

PCR was used to assess the production of cDNA with specific primer sets. This method allows the production of copies of a target DNA sequence from only a few molecules. The PCR reaction mixture contained 2 μl of 10 x KCl reaction buffer (Mg free), 2 μl of 2 mM dNTPs, 1.2 μl of 25 mM $MgCl_2$ (all from Helena Biosciences), 2 μl of 5 mM betaine monohydrate, 0.4 μl of each 10 μM primer (Table 2.1), 9 μl of nuclease free water and finally 0.1 μl of 2.5 U Taq polymerase. 2 μl of each cDNA sample was added to give a final volume of 20 μl. PCR amplification of the cDNA preparation was carried out with a thermal cycler. β-actin cDNA co-amplification was performed during the last 16 cycles of PCR and was used as a control. Depending on the transcripts to be studied, different PCR conditions were adopted and run for between 30 and 40 cycles, finally returning to 4°C for storage (refer to Appendix 1). The PCR products were run on an agarose gel as described above.

For HLA-G specific amplification the primers, G2-5′ and GC-2a, placed at exon 2 and 3′UT regions, respectively, were used. Due to the low number of cells extracted for RNA and, hence, the low amount of cDNA obtained, nested RT-PCR was then undertaken to identify transcripts. The PCR product (1-2 μl) from the initial PCR reaction was used for nested PCR. The following primers used: G257F (exon2)/

HLA-GEx5 (exon5) detecting G1-G6 isoforms; G526F (exon3)/HLA-GEx5 identifying G1, G4 and G5, and G257F/i4b (intron4) detecting the G5 and G6 soluble isoforms (refer to Appendix 1). A JEG-3 cell line was used as a positive control (donated by Professor J Pena, University of Cordoba, Spain).

2.2.8 Statistical analysis

A comparison of the sample means was performed by a Student's *t* test. A value of $p<0.05$ was considered significant.

Chapter III

RESULTS 1: PHENOTYPIC CHARACTERISATION OF DENDRITIC CELL SUBSETS

3.1 Introduction

Dendritic cells are $CD45^+$ BM-derived leucocytes that lack the classical markers specific for T cells (CD3), B cells (CD19/CD20), monocytes (CD14), or NK cells (CD16/CD56) and are defined as a lineage negative (lin^-) cell population, however, they express HLA-DR (Steinman et al., 1979; Van Voorhis et al., 1983; Hart and McKenzie, 1988). The $lin^-HLA\text{-}DR^+$ phenotype distinguishes DCs from other leukocytes. The population of DCs is comprised of a network of morphologically, phenotypically and functionally distinct subsets. The two main subsets identified in APB are MDCs and PDCs (O'Doherty et al., 1994; Robinson et al., 1999). The characterisation of these subsets has only just emerged in the last few years due to their low frequency (0.5 - 1%) and the previous lack of specific markers (Fearnley et al., 1999; Hart, 1997). Original markers of identification included CD11c for MDCs and CD123 for PDCs (O'Doherty et al., 1994). More recently, other markers have been described. They are denoted CD1c and BDCA3 (CD141), expressed on MDCs, and BDCA2 (CD303) and BDCA4 (CD304) both found on PDCs (Dzionek et al., 2000).

The emergence of specific markers for DC subsets, and advances in the isolation techniques such as the introduction of automated immuno-magnetic bead separation (autoMACS) and flow cytometry, have provided a platform on which to study the phenotypic characteristics of MDCs and PDCs. The phenotype of APB DC subsets is well established and major differences have been observed between MDCs and PDCs (O'Doherty et al., 1994). In contrast, the phenotypes of MDCs and PDCs from CB have not been studied extensively. Hence, the aim of this chapter was to identify any differences between CB MDCs and PDCs, and between CB and APB, which may be due to the stage of DC development or maturation, or their existence as distinct entities. To accomplish this, a range of markers were studied on CB MDCs and PDCs, such as molecules to identify and distinguish between the two different DC subsets, and molecules involved in processing and presenting antigen, such as Ii chain, CLIP, HLA-DM and HLA-DR. Markers of immaturity/precursor cells including HLA-G, CD34, CD45RA and CD133 were also studied.

Absolute counting of both DC subsets was initially performed to establish any differences between MDC and PDC prevalence. Subsequently, immunophenotyping was performed by flow cytometry on the two DC subsets following their isolation using the MACS system. Studying the expression of various molecules on DC subsets was important to establish any further differences between MDCs and PDCs, but also any differences between CB and APB.

3.2 Results

3.2.1 Absolute cell counting

Whole blood volumes were used to determine absolute cell counts before isolating DC subsets from either CB or APB. When comparing DC subsets there was a significant difference in absolute counts with higher counts of PDCs (14 ± 4 PDCs/μl) than MDCs (8 ± 3 MDCs/μl) in CB ($p < 0.05$) (Figure 3.1a). In contrast, significantly more MDCs (11 ± 4 MDCs/μl) than PDCs (6 ± 1 PDCs/μl) were observed in the adult setting ($p < 0.05$) (Figure 3.1a).

When comparing CB and APB, CB MDCs (8 ± 3 MDCs/μl) were present in lower numbers than APB MDCs (11 ± 4 MDCs/μl), but this difference was not significant ($p > 0.05$) (Figure 3.1a). However, absolute counts of CB PDCs (14 ± 4 PDCs/μl) were significantly higher than counts for APB PDCs (6 ± 1 PDCs/μl) ($p < 0.05$) (Figure 3.1a). All results are shown as the mean ± SD of five independent experiments.

In terms of percentage, CB MDCs consisted of 36 ± 4% of the DC population and CB PDCs represented 64 ± 3%. The values were inverted in APB, with a mean value of 65 ± 6% for MDCs and 35 ± 6% for PDCs (Figure 3.1b).

3.2.2 DC subset isolation

APB DCs were initially isolated using the 'total DC isolation' technique in which both DC subsets were present. This was replaced following the introduction of

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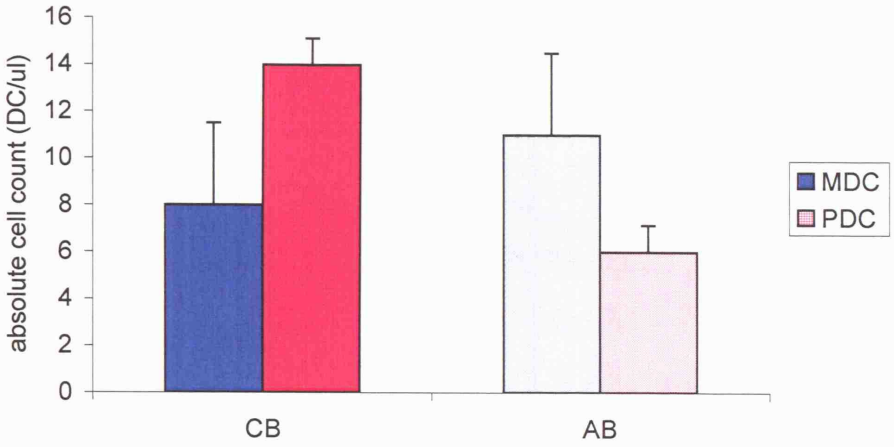

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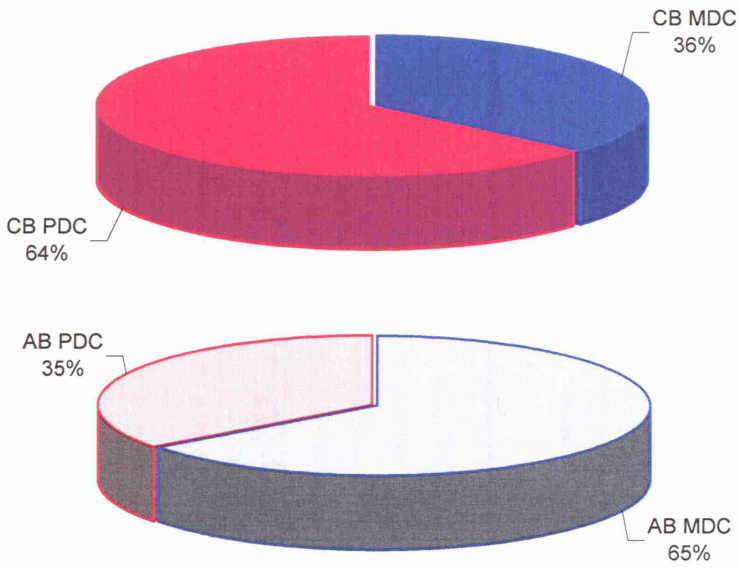


Figure 3.1. The absolute counts of MDCs and PDCs present in CB and APB.

The absolute cell counts of MDCs and PDCs were determined by flow cytometric analysis. This involved incubating cells with and equal volume of flow count fluorospheres after labelling whole blood with DC specific MoAbs. Differences were detected between DC subsets from CB and APB in terms of (a) absolute counts and (b) percentage positive cells (MDC:PDC ratio). The results are the mean of five independent experiments with different donors (n = 5).

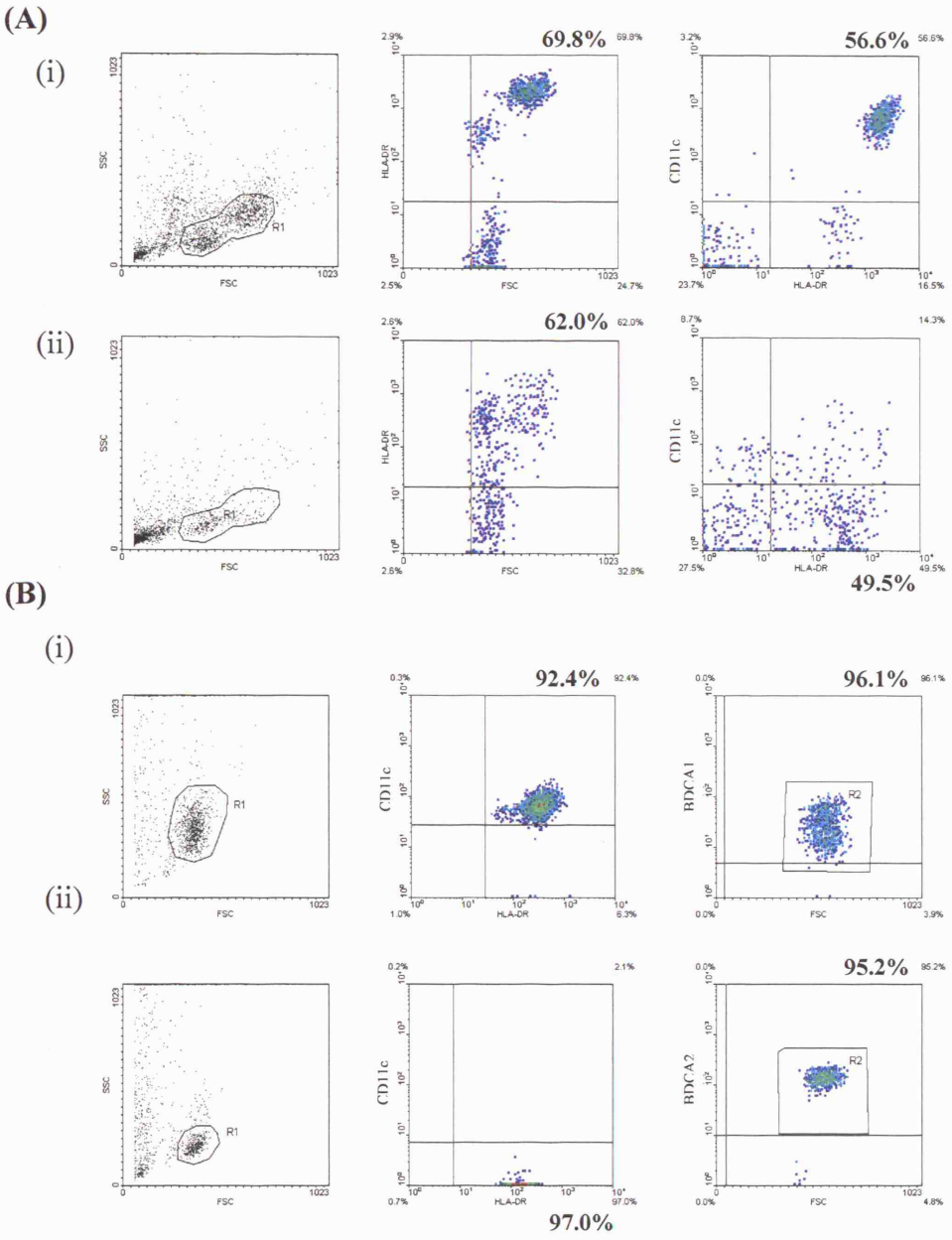


Figure 3.2. The purity of myeloid and plasmacytoid dendritic cells isolated using the magnetic column system.

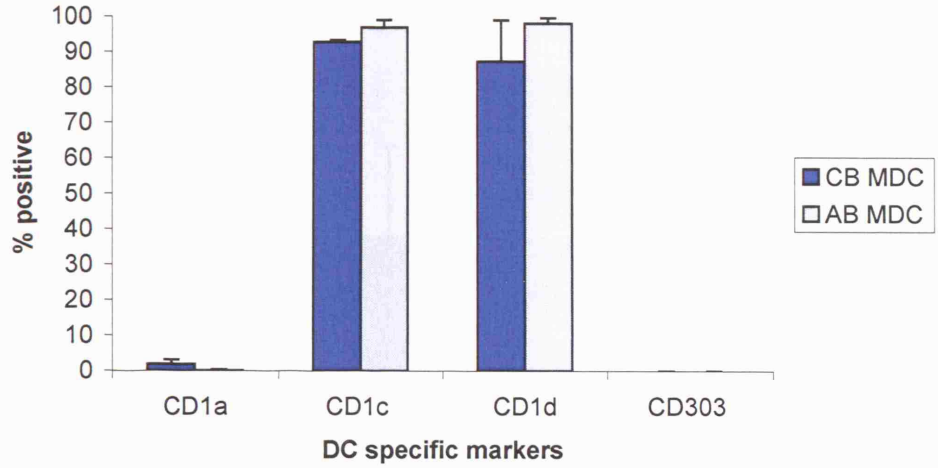
(A) Isolation of (i) MDCs and (ii) PDCs using a manual magnetic cell sorting system following labelling with cell specific MoAbs. Purity achieved was consistently low, as demonstrated by the dotplots. (B) Isolation of (i) MDCs and (ii) PDCs from CB and APB using the autoMACS following labelling with cell specific MoAbs. Purity achieved was consistently above 90% as demonstrated by the dotplots. These dotplots are representative of the images obtained for all experiments.

methods to isolate individual DC subsets based on markers specific for each of the DC subsets. The separation of DCs was initially performed using the manual system of magnetic column cell separation (MACS), obtaining a low purity (50-60%) for both DC subsets (Figure 3.2A). With a combination of operator experience and the introduction of the autoMACS, the purity of MDCs and PDCs dramatically increased to 95% (Figure 3.2B). This automated system also allowed the use of a greater number of MNCs in the initial separation process (400 x 10^6 compared to 200 x 10^6 manually) and increased sensitivity. The purity of the two DC populations was demonstrated by studying the expression of molecules specific for DC subsets, CD1c and CD303 (Figure 3.2 – 3.4). Neither the CD1 or CD303 markers were expressed on the opposite DC subset, showing subset specificity.

Members of the CD1 family of markers, CD1a, c, and d molecules, which are thought to be present on myeloid derived DCs were examined. CD1a was absent on both CB and APB MDCs (Figure 3.3a). As expected, MDCs expressed CD1c, a marker commonly used to identify MDCs (CB MDCs: 93% ± 0.6 and APB MDCs: 97.1% ± 2.2) (Figure 3.3a) with similar levels of expression (CB MDCs MFI: 74.2 ± 13.2 and APB MDCs MFI: 89 ± 9) (Figure 3.4a). CD1d was also present on the majority of CB MDCs (87.5% ± 11.7) and APB MDCs (98.3% ± 1.5) (Figure 3.3a) with a high level of expression (CB MDCs MFI: 113.7 ± 77.3 and APB MDCs MFI: 185 ± 68) (Figure 3.4a). There was no significant difference in the expression of CD1 molecules between CB and APB MDCs. PDCs from CB and APB did not express CD1 molecules on their surface (Figures 3.3 and 3.4) showing exclusivity of this family for MDCs.

The level of expression of CD303, a marker for PDC identification, was similar between CB PDCs (MFI: 151.1 ± 0.5) (Figure 3.3b) and APB PDCs (MFI: 163.4 ± 58.9) (Figure 3.4b). MDCs did not express CD303 (Figures 3.3a and 3.4a).

(a)



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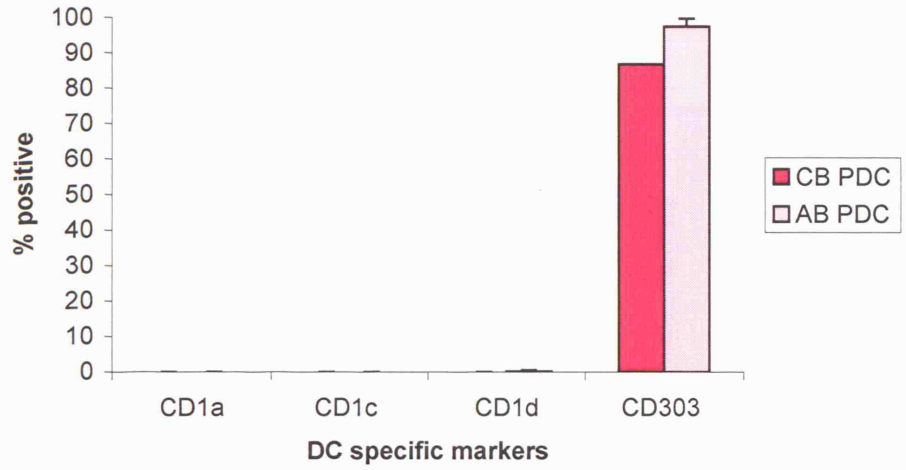
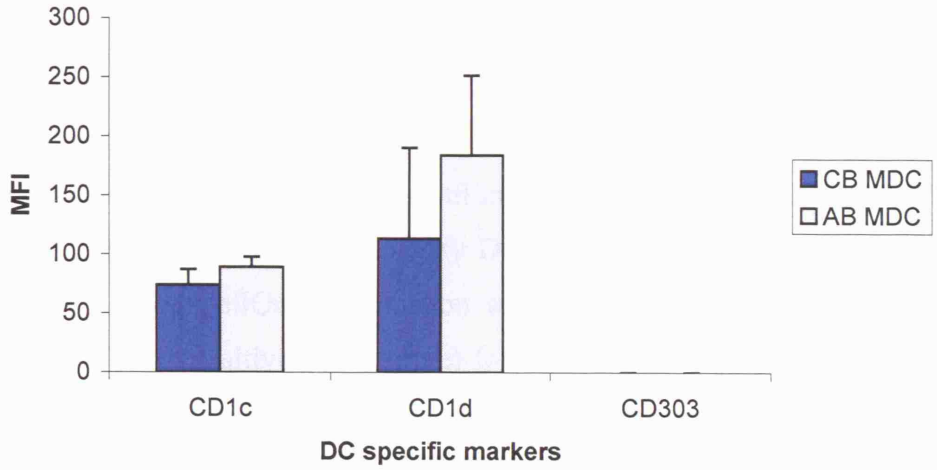


Figure 3.3. Percentage expression of CD1 and CD303 on MDCs and PDCs present in CB and APB.

The expression of the markers, CD1 and CD303 was assessed to determine the specificity of (a) MDCs for CD1 and (b) PDCs for CD303. This was achieved by flow cytometric analysis after immunophenotyping was performed. The results show the mean ± SD of three independent experiments (n = 3).

(a)



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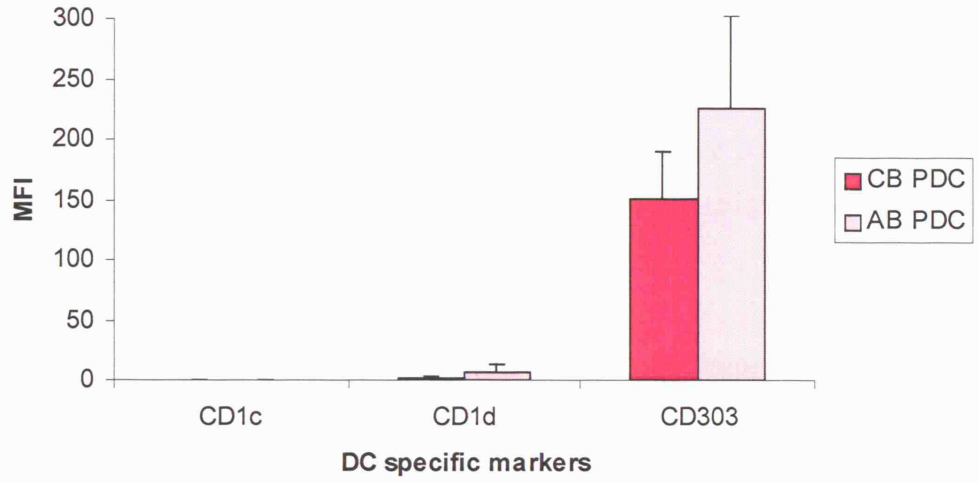

Figure 3.4. Levels of expression of CD1 and CD303 on MDCs and PDCs present in CB and APB.

The level of expression of the markers, CD1 and CD303 was assessed to determine the specificity of (a) MDCs for CD1 and (b) PDCs for CD303. This was achieved by flow cytometric analysis after immunophenotyping was performed. The results show the mean ± SD of three experiments (n = 3).

3.2.3 Immunophenotyping

Following isolation from CB and APB, immunophenotyping of the MDCs and PDCs was performed with a panel of MoAbs (refer to Appendix 1). The molecules were selected according to their reported differential expression on individual DC subsets. Depending on the marker, membrane labelling and/or intracellular labelling was performed. Surface expression of all molecules was examined co-staining with CD1c or CD303 and HLA-DR to verify DC identity. At least 5,000 events were acquired using the CellQuest acquistion software by flow cytometry. Both the percentage of cells positive (% positive) for a particular molecule and the level of expression denoted as MFI (for percentages above 10%), were determined (see Appendix 2). The level of expression was not determined when cells were negative, or minimally expressed a particular marker (1-10% positive). All results are shown as the mean ± SD of at least three individual experiments.

3.2.3.1 Expression of HLA molecules

The HLA molecules studied by flow cytometry included the classical HLA Class I (HLA-ABC), the non-classical HLA-E and HLA-G (Figure 3.5 and 3.6), and the HLA Class II molecules, HLA-DR, HLA-DQ and HLA-DP (Figure 3.8 and 3.9).

3.2.3.1.1 Classical HLA Class I molecules

The results showed that both CB and APB DC subsets expressed HLA Class I molecules. The percentage of cells positive for HLA Class I was similar between CB MDCs (98.3% ± 0.8) and CB PDCs (99.9% ± 0.3) (Figure 3.5a), and between APB MDCs (98.9% ± 1.5) and APB PDCs (99.8% ± 0.2) (Figure 3.5b). However, there were differences in the level of expression, with CB MDCs (MFI: 312.9 ± 8.2) expressing significantly higher levels of HLA Class I than CB PDCs (MFI: 103.4 ± 1.9) (Figure 3.6a). This pattern of expression was repeated for APB MDCs (MFI: 513.8 ± 184) and APB PDCs (MFI: 287 ± 168) (Figure 3.6b). The overall level of HLA Class I expression was lower on CB PDCs than on CB MDCs, and on CB compared to APB.

3.2.3.1.2 Nonclassical HLA Class I molecules

The expression of the non-classical HLA Class I molecules, HLA-G and HLA-E, was also studied (Figures 3.5 and 3.6).

HLA-G: A low percentage of CB MDCs (1.6% ± 1.2) and PDCs (1% ± 1.3) (n = 10) expressed surface HLA-G, but there was no expression on APB MDCs or PDCs (n = 7) (Figure 3.5). However, a high percentage of cells were positive for intracellular HLA-G, with an overall higher expression in CB than in APB (Figure 3.5). CB MDCs expressed a higher percentage and MFI of intracellular HLA-G (61.3% ± 31.6 and MFI: 22.5 ± 12.1) compared to CB PDCs (48.7% ± 42.1; MFI: 12.1 ± 7.9) (Figures 3.5a and 3.6a). This differential expression pattern was repeated with APB MDCs (17.6% ± 13.3, MFI: 16.7 ± 4.3) and APB PDCs (10.9% ± 5.5, MFI: 5.6 ± 6.6) but with a generally lower percentage of positive cells (Figure 3.5b and 3.6b).

As CB DCs expressed surface HLA-G, further investigation to detect the various HLA-G isoforms in DC subsets was performed by nested RT-PCR using cDNA. JEG-3 was used as the positive control. The PCR products of the HLA-G isoforms (HLA-G1 to -G6) were studied and gel images produced as represented in Figure 3.7. Membrane HLA-G1 and soluble HLA-G5 mRNA were present in all CB MDCs (n = 9) and PDCs (n = 6) tested. CB MDCs expressed higher levels of HLA-G1 mRNA, compared to JEG-3 represented by the high intensity of the bands on the gel (Figure 3.7). HLA-G3 was detected in two CB PDC samples at the same intensity as JEG-3.

APB DCs also expressed mRNA transcripts, but only six out of ten MDCs were positive for HLA-G1 and G5. Four out of ten APB PDCs presented with HLA-G mRNA. In three of the four PDC samples, HLA-G1 and G5 were detected and HLA-G3 was also observed in addition, in one remaining sample. The intensity of expression was low in APB DCs and CB PDCs compared to JEG-3.

HLA-E: No surface HLA-E expression was detected on either CB or APB DC subsets (Figure 3.5). Intracellular HLA-E was detected on a lower percentage of CB

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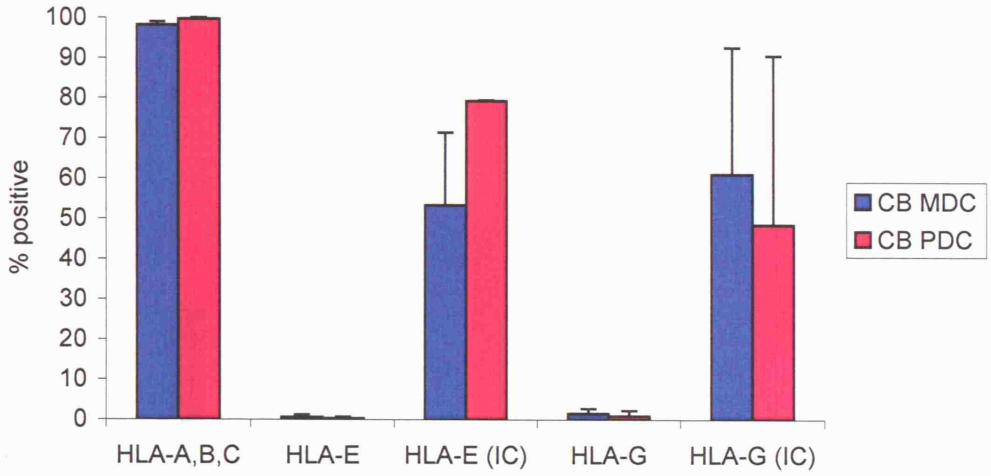

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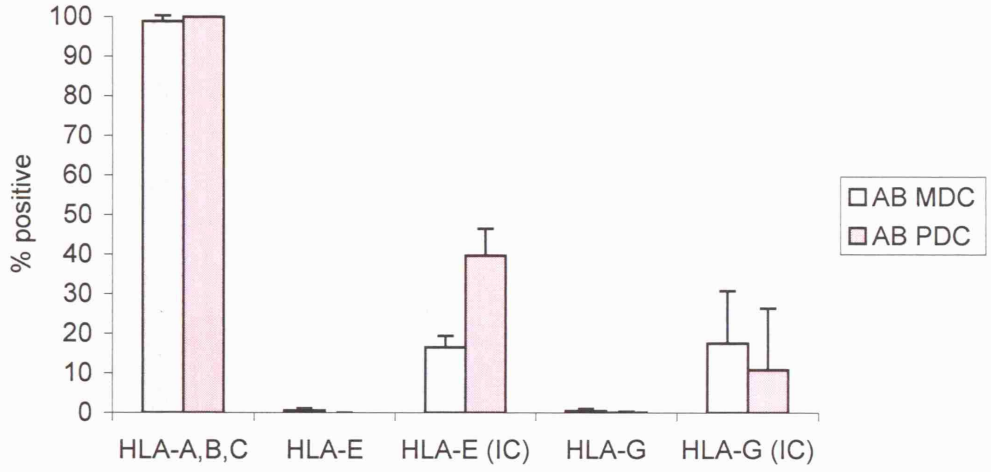
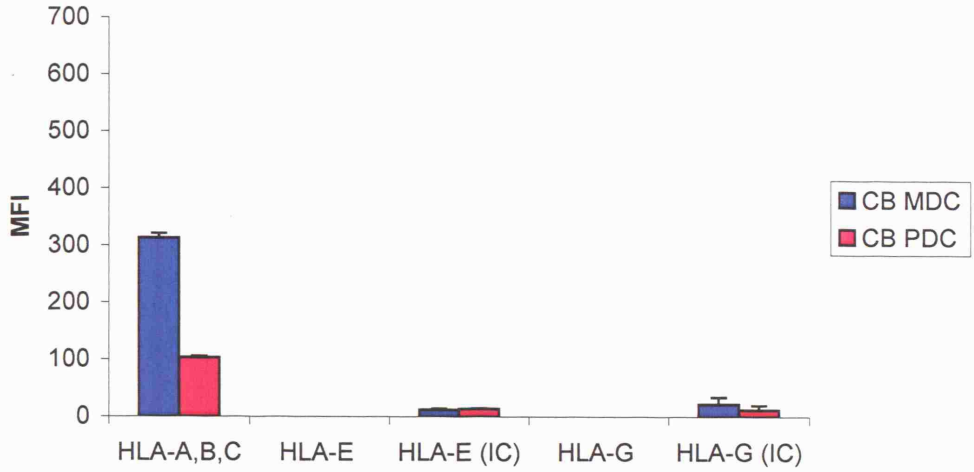


Figure 3.5. Percentage expression of HLA Class I molecules on MDCs and PDCs present in CB and APB.

Immunophenotyping was performed, studying MHC Class I molecule expression on the DC subsets from CB and APB. The cells were labelled with HLA-ABC (n = 3), HLA-E (n = 3), or HLA-G (n = 10), and results obtained by flow cytometry of (a) CB and (b) APB MDCs and PDCs.

(a)



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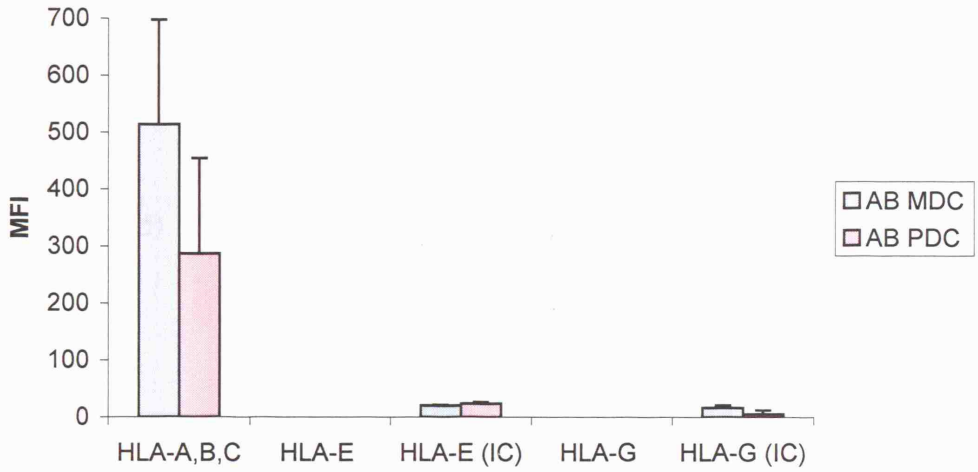


Figure 3.6. Levels of expression of HLA Class I molecules on MDCs and PDCs present in CB and APB.

The level of expression of the DC subsets was also determined if a positive result had been obtained. This was determined from the MFI value. The cells were labelled with HLA-ABC (n = 3), HLA-E (IC) (n = 3), or HLA-G (IC) (n = 10), and results obtained by flow cytometry of (a) CB and (b) APB MDCs and PDCs.

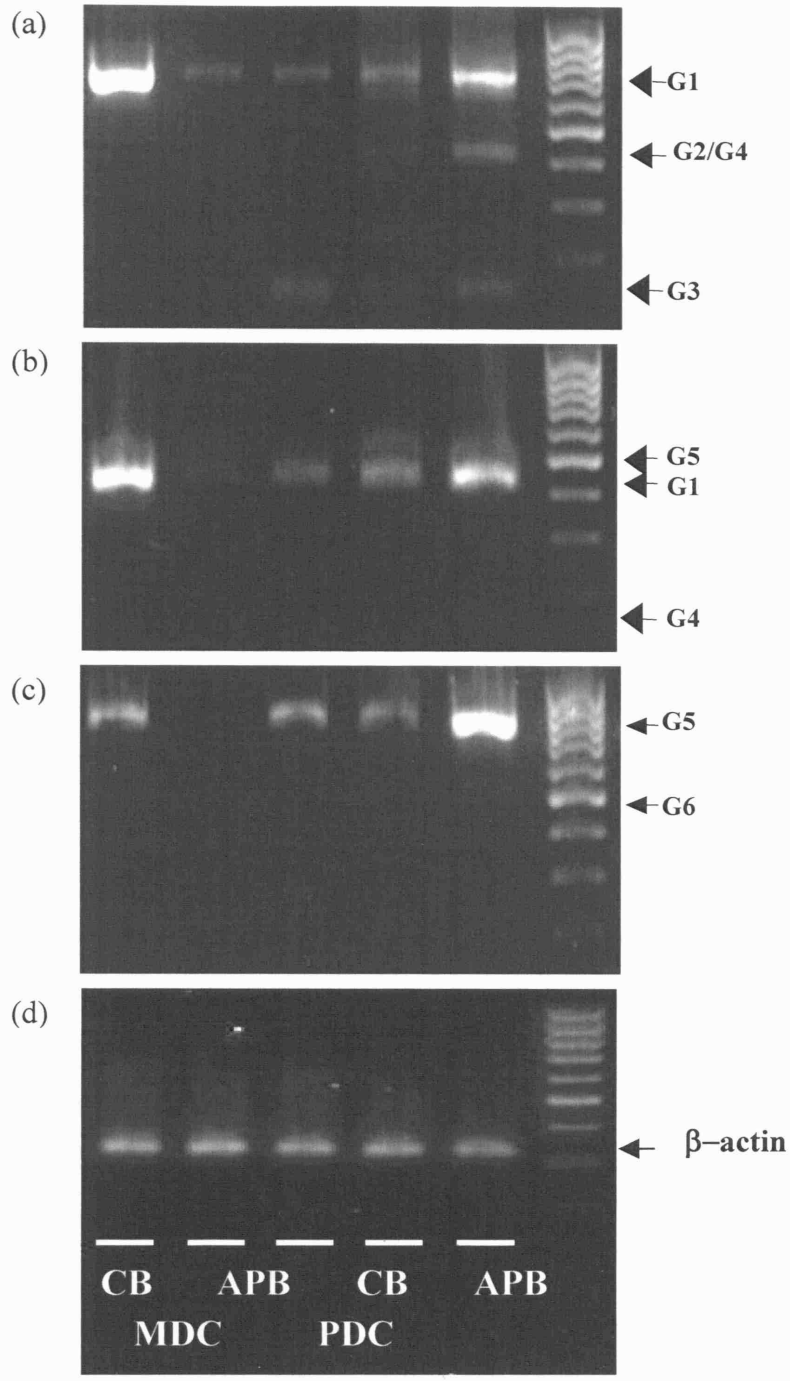


Figure 3.7. Gel electrophoresis images of HLA-G isoforms present at the mRNA level in MDCs and PDCs present in CB and APB.

RT-PCR was undertaken to study the expression of the nonclassical HLA-G molecule in the DC subsets from CB and APB. Specific primers were used to identify the different isoforms (HLA-G1-G6) (a - c). β-actin was the internal control (d) and JEG-3 the positive control (a – d).

MDCs (53.4% ± 18.2) than CB PDCs (79.5% ± 0.1) (Figure 3.5a). Intracellular HLA-E expression in APB MDCs (16.6% ± 2.9) was lower than in APB PDCs (39.8% ± 6.8) (Figure 3.5b). The level of expression of intracellular HLA-G was similar between CB MDCs (MFI: 12.5 ± 1.2) and CB PDCs (MFI: 13.9 ± 0.8) (Figure 3.6a), and the pattern was reciprocated in APB MDCs (MFI: 20.8 ± 0.7) and APB PDCs (MFI: 23.9 ± 2.8) (Figure 3.6b). The results are shown as the mean ± SD of three independent experiments.

3.2.3.1.3 Expression of HLA Class II molecules

The expression of HLA-DR, HLA-DQ and HLA-DP molecules was also studied by flow cytometry (Figure 3.8 and 3.9).

HLA-DR: The expression of surface HLA-DR was similar between CB MDCs (95.7% ± 4.4) and CB PDCs (96.2% ± 3) and between APB MDCs (99.1% ± 0.9) and APB PDCs (96.9% ± 3.9) (n = 13) (Figure 3.8). However, the level of expression was significantly higher on MDCs (CB MFI: 211 ± 38.8; APB MFI: 433 ± 241) compared to PDCs (CB MFI: 94.8 ± 55.2; APB MFI: 183 ± 101) (Figure 3.9). The results showed an overall diminished level of expression in the CB DC subsets compared to APB DCs, thus showing a significant difference between CB and APB DC subsets ($p < 0.05$) (Figure 3.9). CB MDCs (99.8% ± 0.2) and CB PDCs (98.9% ± 0.4) were highly positive for intracellular HLA-DR (Figure 3.8a). APB MDCs (98.8% ± 1.6) and APB PDCs (99.5% ± 0.4) were also positive for intracellular HLA-DR (Figure 3.8b). Levels of expression on CB MDCs (MFI: 238.6 ± 74.1) were significantly higher compared to CB PDCs (MFI: 73.5 ± 27.9) ($p < 0.05$) (Figure 3.9a). The level of expression on APB MDCs (MFI: 522.4 ± 148) was five-fold higher compared to APB PDCs (MFI: 104.5 ± 57) (Figure 3.9b). All results are shown as the mean ± SD of six individual experiments. These results confirmed the similarity between MDCs and PDCs in the percentage of cells positive for HLA-DR on the cell surface and intracellularly. However, the level of expression, both at the cell surface and intracellularly was higher in MDCs than in PDCs, with an overall diminished expression in CB DCs.

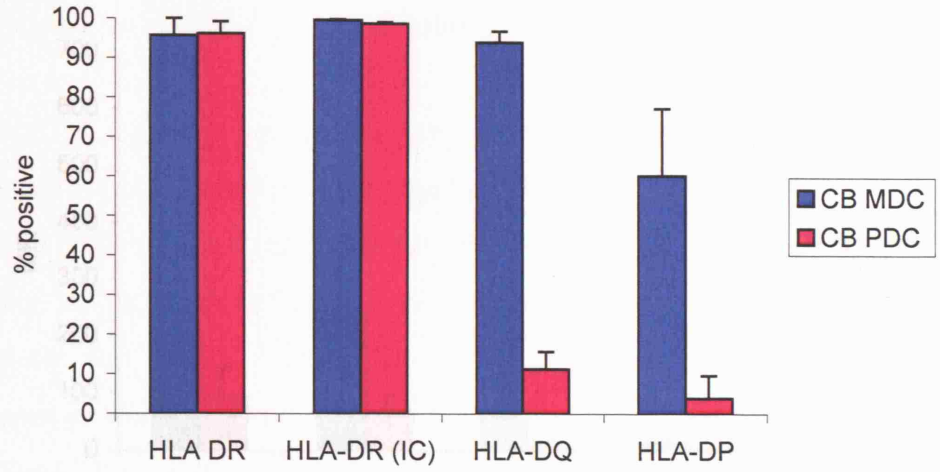
HLA-DQ: HLA-DQ molecules were expressed by both the CB and APB DC subsets, but the percentage of cells positive for each subset differed. A substantially higher number of CB MDCs (94.1% ± 2.8) were positive for HLA-DQ (Figure 3.8a) than CB PDCs (11.4% ± 4.3). This pattern of HLA-DQ expression was repeated with APB MDCs (96.5% ± 1.9) and APB PDCs (27.8% ± 7) (n = 6) (Figure 3.8b). Hence, the level of expression on CB MDCs (MFI: 60.9 ± 42.8) was higher compared to CB PDCs (MFI: 8.8 ± 9.7) (Figure 3.9a), repeated on APB MDCs (MFI: 64 ± 18) and APB PDCs (MFI: 10 ± 4) (Figure 3.9b). In summary, both the percentage of cells positive for HLA-DQ and the level of expression were higher on MDCs than PDCs.

HLA-DP: The percentage of CB MDCs expressing HLA-DP was lower (67.9% ± 19.1) than those expressing HLA-DR and HLA-DQ (n = 5), but significantly higher than the percentage expression on CB PDCs (4% ± 5.7) (Figure 3.8a). The expression of HLA-DP was also significantly higher on APB MDCs (94.1% ± 4) than on APB PDCs (18.4% ± 26.7) (Figure 3.8b). The level of expression of HLA-DP on APB MDCs (MFI: 81.4 ± 6) and APB PDCs (MFI: 19 ± 32) (Figure 3.9b) was elevated compared to the CB MDCs (MFI: 17.3 ± 5.2). Overall, MDCs expressed several-fold higher levels of HLA-DP than PDCs, and CB expressed lower numbers and levels of HLA-DP compared to APB. The results are shown as the mean ± SD of five independent experiments.

3.2.3.2 Expression of costimulatory molecules and maturation markers

The expression of the costimulatory molecules, CD80 and CD86, was assessed. The percentage of CB MDCs (90.3% ± 1.5) positive for CD86 was significantly higher than the percentage on CB PDCs (6.2% ± 3.2) ($p < 0.05$). This difference in CD86 expression was repeated on APB MDCs (89.5% ± 5.7) and APB PDCs (11.5% ± 6.4) ($p < 0.05$). The level of expression on CB MDCs (MFI: 77.8 ± 16.5) was similar to APB MDCs (MFI: 68.1 ± 40.1). Both CD80 and CD83 expression was absent on MDCs and PDCs present in CB and APB. The results above are the mean ± SD of three and nine independent experiments for CB and APB, respectively.

(a)



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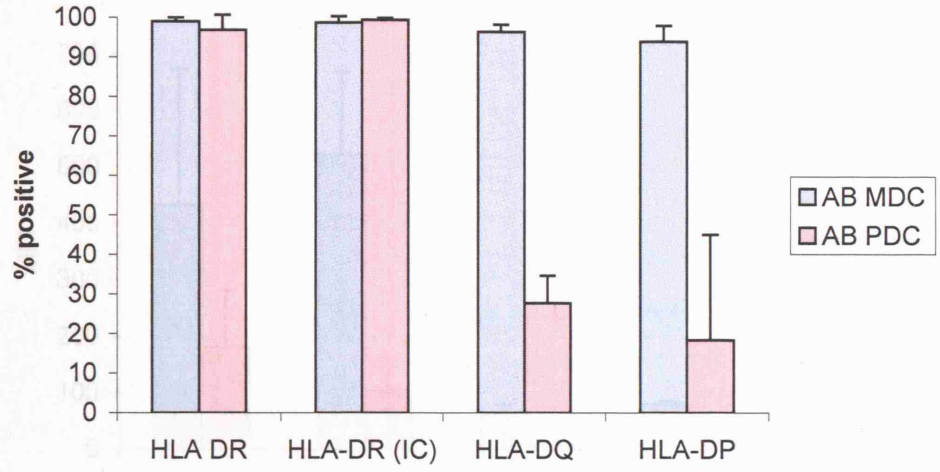
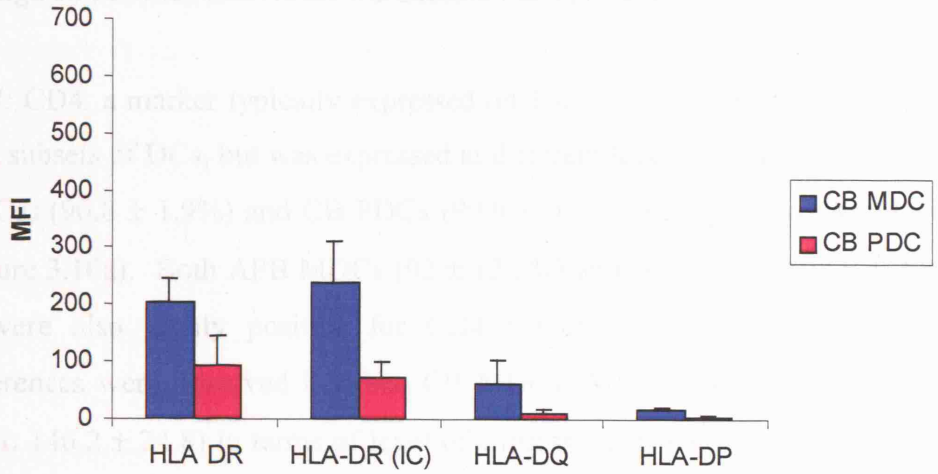


Figure 3.8. Percentage expression of HLA Class II molecules by MDCs and PDCs present in CB and APB.

Immunophenotyping was performed, studying MHC Class II molecule expression on the (a) CB and (b) APB MDCs and PDCs. The cells were labelled with HLA-DR (surface; n = 13, and intracellular; n = 6), HLA-DQ (n = 6) or HLA-DP (n = 5), and results obtained by flow cytometric analysis. The results are expressed as percentage of positive cells for a particular marker.

(a)



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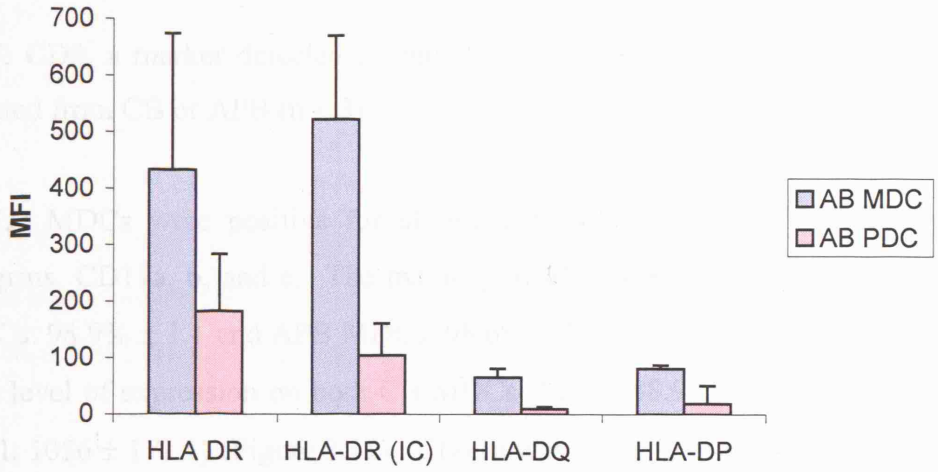


Figure 3.9. Levels of expression of HLA Class II molecules by MDCs and PDCs present in CB and APB.

Immunophenotyping was performed, studying MHC Class II molecule expression on (a) CB and (b) APB MDCs and PDCs. The cells were labelled with HLA-DR (surface; n = 13, and intracellular; n = 6), HLA-DQ (n = 6) or HLA-DP (n = 5), and results obtained by flow cytometric analysis. The results show the level of expression of each molecule and are expressed as the mean fluorescence intensity (MFI).

3.2.3.3 Expression of DC non-specific markers on DCs

The expression of other markers present on various leucocytes, but known to distinguish between individual DC subsets was also studied (Figure 3.10 and 3.11).

CD4: CD4, a marker typically expressed on T cells and monocytes, was present on both subsets of DCs, but was expressed at different levels. A high percentage of CB MDCs (90.8 ± 1.9%) and CB PDCs (99.9 ± 0.1%) were positive for CD4 (n = 5) (Figure 3.10a). Both APB MDCs (92 ± 13.8%) and APB PDCs (98.6 ± 2.8%) (n = 4) were also highly positive for CD4 (Figure 3.10b). However, significant differences were observed between CB MDCs (MFI: 77.4 ± 11.1) and CB PDCs (MFI: 146.2 ± 24.8) in terms of level of expression ($p < 0.05$) (Figure 3.11a). APB MDCs (MFI: 205 ± 81) also expressed significantly lower levels of CD4 than APB PDCs (MFI: 510 ± 131) ($p < 0.05$) (Figure 3.11b). There was an overall lower level of expression on CB MDCs and PDCs compared to APB.

CD8: CD8, a marker detected on murine DCs, was absent on human DC subsets isolated from CB or APB (n = 3).

CD11: MDCs were positive for all members of the CD11/CD18 family of β2 integrins, CD11a, b, and c. The majority of MDCs were positive for CD11a (CB MDCs: 98.9% ± 1.1 and APB MDCs: 98.6% ± 1.7) (Figure 3.10a and 3.10b) with a high level of expression on both CB MDCs (MFI: 688.9 ± 219.8) and APB MDCs (MFI: 1056 ± 171.1) (Figure 3.11). CD11a was the only molecule expressed by CB and APB PDCs. A high percentage of CB PDCs (100% ± 0) and APB PDCs (98.6% ± 1.0) were positive for CD11a (Figure 3.10). The level of expression was reduced on CB PDCs (MFI: 296.7 ± 0.9) in comparison to APB PDCs (MFI: 613 ± 77.3) (Figure 3.11).

CD11b was expressed on a lower percentage of CB MDCs (36.2% ± 18.2) and APB MDCs (48.6% ± 12.5) (Figure 3.10a). This reduction was also reflected in level of expression on CB MDCs (MFI: 9.6 ± 13.5) and APB MDCs (MFI: 13 ± 12) (Figure 3.11a). CB MDCs (99% ± 0.8) and APB MDCs (95% ± 1.8) were also positive for

CD11c (Figure 3.10a) with a higher level of expression on CB MDCs (MFI: 100 ± 0.5) compared to APB MDCs (MFI: 47 ± 19) (Figure 3.11a). The results using CB are shown as the mean ± SD of three independent experiments but varied for CD11a (n = 3), CD11b (n = 9) and CD11c (n = 13) studies on APB.

CD13, CD14 and CD33: MDCs from both CB and APB were positive for CD13 (CB: 10.1 ± 13.7%; APB: 24.1% ± 9.5), CD14 (CB: 38.2% ± 20.7; APB: 28.7% ± 7.3) and CD33 (CB: 92.1% ± 3.2; APB: 96.4% ± 4) (Figure 3.10), which are myeloid markers. The level of expression of CD13 was low on CB MDCs (MFI: 4.1 ± 4) and APB MDCs (MFI: 9.8 ± 4.5) (Figure 3.11). There was a higher level of CD14 expression on CB MDCs (MFI: 57.3 ± 86.7) compared to APB MDCs (MFI: 10 ± 2) (Figure 3.11a and 3.11b). CD33 was expressed at lower levels by CB MDCs (MFI: 137.8 ± 74.1) than by APB (MFI: 268.7 ± 69.4) (Figure 3.11). Conversely, PDCs from CB or APB were negative for CD13 and CD14, but both CB PDCs (17.1% ± 14.2) and APB PDCs (46.1% ± 21.7) were positive for CD33 (Figure 3.10). The results are shown as mean ± SD of four independent experiments.

CD34: CD34, a HSC marker, was present on a low percentage of CB MDCs (1.3% ± 0.9), but on a much higher percentage of CB PDCs (16.8% ± 14.5) (Figure 3.10a). There was no expression of CD34 on either APB MDCs or PDCs (Figure 3.10b). The results are shown as the mean ± SD of four independent experiments.

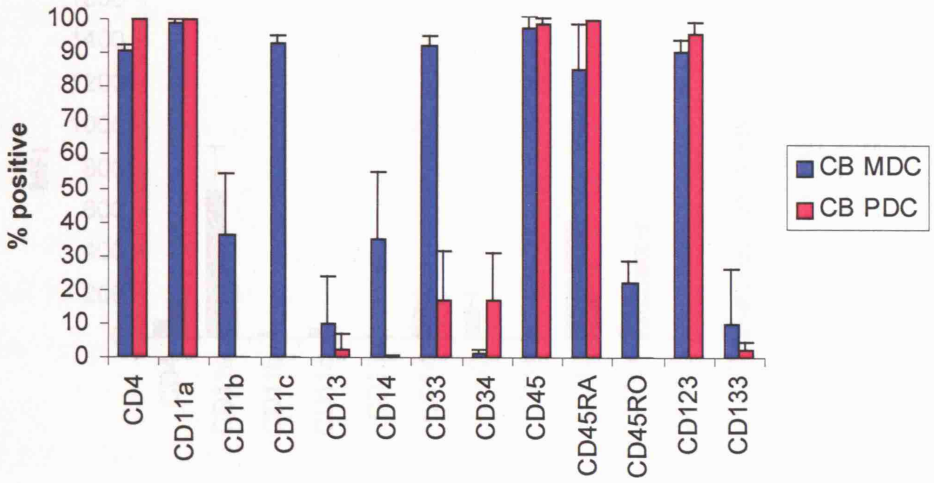
CD45: The pan leucocyte antigen, CD45, was expressed by both CB (n = 6) and APB DC subsets (n = 4). The percentage of CB cells positive for CD45 was similar between CB MDCs (97.8% ± 3.1) and CB PDCs (99.1% ± 1.7) (Figure 3.10a). The percentage of APB cells positive for CD45 was similar between MDCs (95.8% ± 5.4) and PDCs (99.0% ± 1.1) (Figure 3.10b). The level of expression was higher on CB MDCs (MFI: 565 ± 358.8) than on CB PDCs (MFI: 195.4 ± 153.6) (Figure 3.11a), increased further with APB MDCs (MFI: 734.3 ± 190.7) and PDCs (MFI: 560 ± 107.4) (Figure 3.11b).

CD45RA and CD45RO: The expression of naïve (RA) and memory (RO) isoforms of CD45 was assessed on DCs present in CB and APB (n = 4). CB MDCs (85.2% ± 13.5) and CB PDCs (100% ± 0) were highly positive for CD45RA (Figure 3.10a). APB MDCs expressed CD45RA (48.9% ± 34.8) at a significantly lower percentage than APB PDCs (98. %4 ± 1.9) (n = 6), which were solely positive for CD45RA. The level of expression of CD45RA on CB MDCs (MFI: 65.5 ± 46.5) was significantly lower than on CB PDCs (MFI: 563.6 ± 123.7) (Figure 3.11a). The level of expression of CD45RA on APB MDCs (MFI: 24 ± 16) was significantly lower than the expression on APB PDCs (MFI: 493 ± 453) (Figure 3.11b).

CB MDCs were also positive for CD45RO (24.9% ± 15) (n = 6) but CB PDCs did not express this molecule (Figure 3.10a). APB MDCs expressed a higher percentage of CD45RO (51% ± 24.5) (n = 6) compared to CB MDCs (Figure 3.10b). APB PDCs (n = 6) did not express CD45RO (Figure 3.10b). The level of expression of CD45RO (7.2 ± 4.4) on CB MDCs was significantly lower than expression of CD45RO (MFI: 19.8 ± 13.9) (Figure 3.11a). Overall, CB DCs preferentially expressed CD45RA.

CD123: CD123 (IL-3R) is a marker used to identify PDCs following isolation and differentiate them from the MDC subset. Both CB MDCs (90.9% ± 3.6) and CB PDCs (96.8% ± 2.6) were highly positive for CD123 as shown by percentage positive cells (Figure 3.10a). However, the percentage of APB cells positive for CD123 was lower for MDCs (75.3% ± 15.3) than for PDCs (99.2% ± 0.6) (Figure 3.10b). CB PDCs expressed significantly higher levels of CD123 (MFI: 1177.0 ± 280.5) than CB MDCs (MFI = 73.1 ± 21.3) (Figure 3.11a). APB PDCs also expressed high levels of CD123 (MFI: 750.5 ± 319.3) with low expression on APB MDCs (MFI: 39.1 ± 11.8) (Figure 3.11b). Overall, CD123 was highly expressed on PDCs compared to MDCs and on CB DCs compared to APB DCs. The results show the mean ± SD of seven and five independent experiments for CB and APB DC subsets, respectively.

(a)



(b)

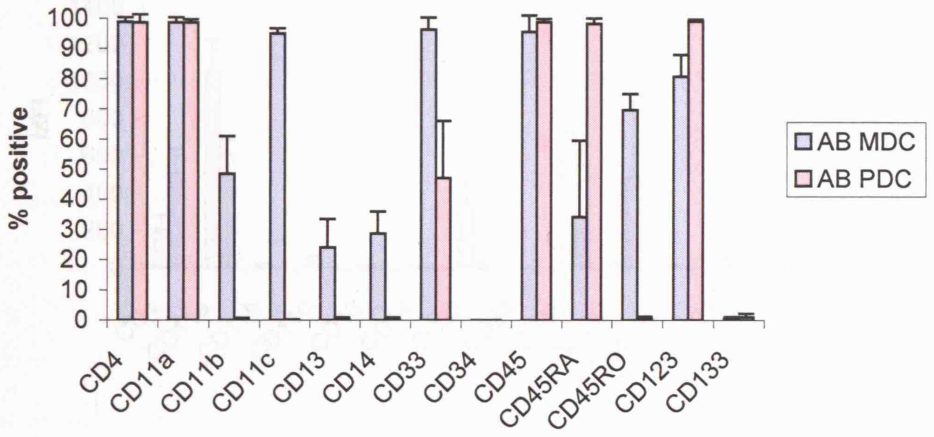
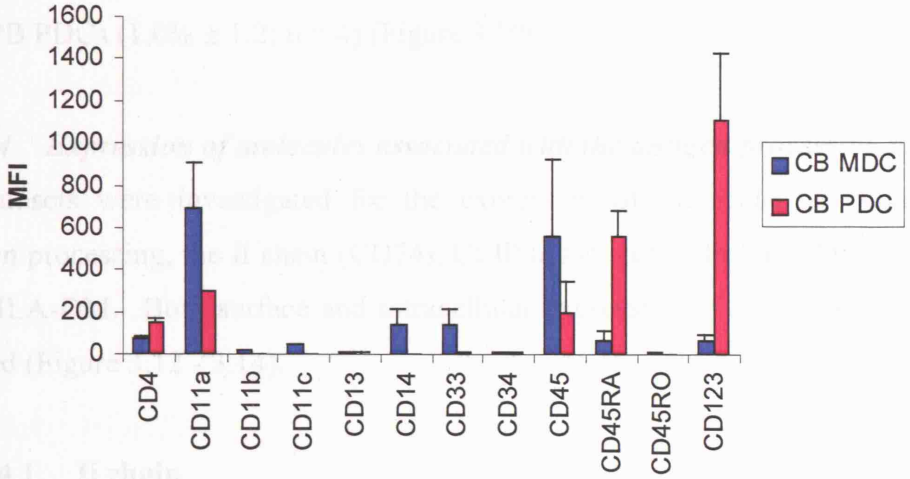


Figure 3.10. Percentage expression of CD molecules on MDCs and PDCs present in CB and APB.

The expression of specific markers to distinguish between MDCs and PDCs on (a) CB and (b) APB, was studied by flow cytometric analysis after immunophenotyping was performed. The results are shown as the mean ± SD of at least three independent experiments.

(a)



(b)

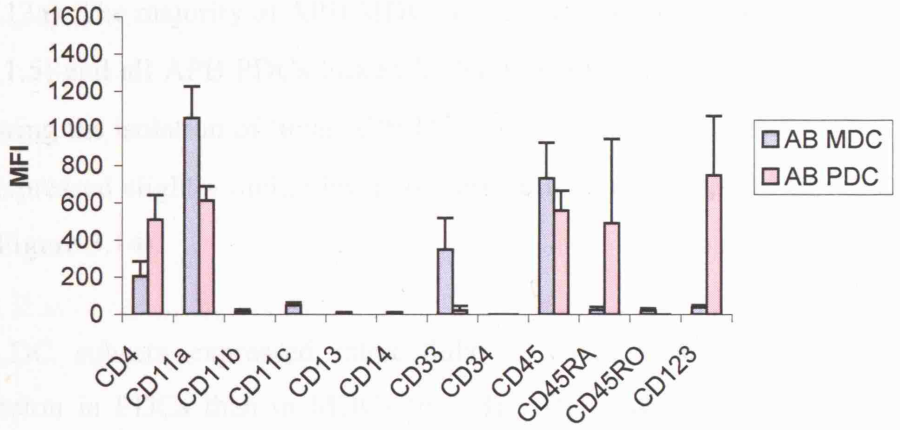


Figure 3.11. Levels of expression of CD molecules on MDCs and PDCs present in CB and APB.

The expression of specific markers to distinguish between MDCs and PDCs in (a) CB and (b) APB, was studied by flow cytometric analysis after immunophenotyping was performed. The results are shown as the mean ± SD of at least three independent experiments.

CD133: CD133, a marker for primitive progenitors, was present on CB MDCs (9.8% ± 16.3; n = 9) at a higher percentage than on CB PDCs (2.3% ± 2.3; n = 6) (Figure 3.10a). CD133 was absent on APB MDCs (n = 4) and minimally expressed on APB PDCs (1.0% ± 1.2; n = 4) (Figure 3.10b).

3.2.3.4 Expression of molecules associated with the antigen processing pathway

DC subsets were investigated for the expression of molecules associated with antigen processing, the Ii chain (CD74), CLIP associated with HLA-DR (CerCLIP), and HLA-DM. Both surface and intracellular expression of these molecules were studied (Figure 3.12 - 3.14).

3.2.3.4.1 Ii chain

The cell surface expression of the Ii chain was detected on CB MDCs (46.2% ± 18.6; MFI: 16.8 ± 5.1) and to a lesser extent on CB PDCs (9.5% ± 6) (Figures 3.12a and 3.13a). The majority of APB MDCs were negative for Ii chain (12.7% ± 2; MFI: 6.5 ± 1.5) and all APB PDCs lacked Ii chain expression (Figures 3.12b and 3.13b). Following the isolation of 'total APB DCs', the results showed that MDCs (12.7% ± 1.4) expressed slightly higher levels of surface Ii chain compared to PDCs (8.9% ± 1.7) (Figure 3.14).

Both DC subsets expressed intracellular Ii chain with a several fold higher expression in PDCs than in MDCs (n = 8). The percentage of cells positive for intracellular Ii chain were similar between CB MDCs (83.3% ± 14) and CB PDCs (93.8 ± 5.3%) (Figure 3.12a) and between APB MDCs (91.6% ± 12.4) and APB PDCs (98.9% ± 1.4) (Figure 3.12b). The level of expression was significantly higher in CB PDCs (MFI: 642.8 ± 281) compared to CB MDCs (MFI: 254.5 ± 238.6) (Figure 3.13a). There was also a significant increase in the levels of expression of Ii chain in APB PDCs (MFI: 894 ± 450) compared to APB MDCs (MFI: 274.1 ± 252) ($p < 0.05$) (Figure 3.13b). The same pattern was observed for 'total APB DCs' with the expression higher in PDCs (57.6% ± 6.8; MFI: 224 ± 26.6) than in MDCs (22.8% ± 1.7; MFI: 51.2 ± 2.9) (Figure 3.14).

3.2.3.4.2 CLIP

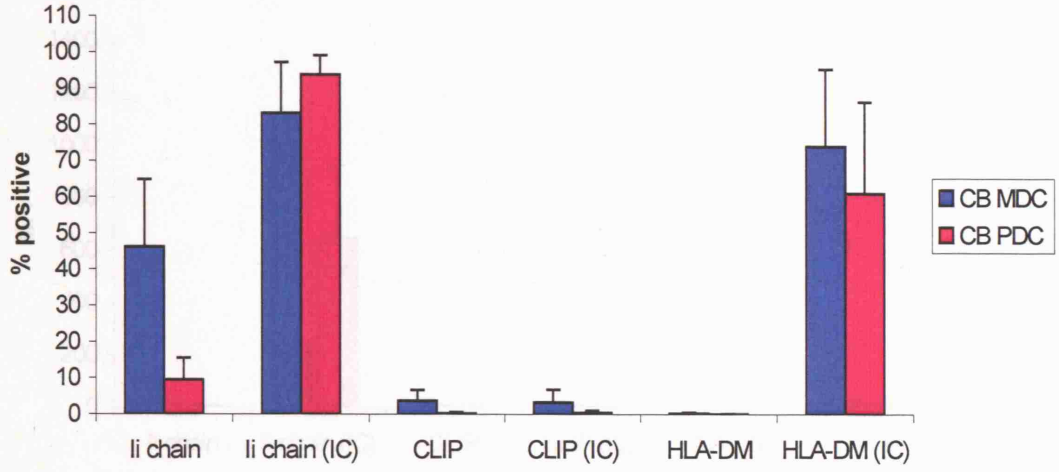
Both surface and intracellular expression of the MHC-II:CLIP complex was exclusive to the MDC subset, with cells from CB expressing a three-fold lower percentage of CLIP (surface: 3.7% ± 3 and intracellular: 3.3% ± 3.5) than APB MDCs (surface: 10.5% ± 7.6 and intracellular: 12.3% ± 15.2) (Figure 3.12a and 3.12b). The results were similar for 'total APB DCs', but the percentage of expression of CLIP doubled with MDCs (surface: 23.5% ± 20.3 and intracellular: 22.9% ± 16.4) and APB PDCs also expressed low levels of surface (6.7% ± 7.8) and intracellular (3.5% ± 3.2) CLIP (Figure 3.14).

3.2.3.4.3 HLA-DM

Surface HLA-DM was not expressed by either CB MDCs or PDCs. However, both CB MDCs (74.1% ± 21.3) and CB PDCs (61.2% ± 25.3) expressed intracellular HLA-DM (Figure 3.12a). APB MDCs (84.7% ± 18.7) and APB PDCs (74.9% ± 27.7) expressed a slightly higher percentage of intracellular HLA-DM compared to CB DCs (Figure 3.12a and 3.12b). The same pattern was observed with the level of expression, where CB MDCs expressed higher levels of HLA-DM (MFI: 30.9 ± 9) than CB PDCs (MFI: 16.1 ± 4.8). The level of expression was again higher in APB MDCs (MFI: 51.6 ± 32.1) and PDCs (MFI: 28.9 ± 0.3) compared to CB (Figure 3.13). The investigation of 'total APB DCs' (n = 12) showed fewer MDCs (31.3% ± 20.2) positive for HLA-DM, compared to PDCs (PDCs: 65% ± 29.9) (Figure 3.14), a result which was in contrast to that of separated DCs.

As well as immunophenotyping data, confocal microscopy images were also obtained for HLA-DM. The areas where there was a high intensity of surface HLA-DR (green) and intracellular HLA-DM (red) was observed in close proximity. In MDCs, both HLA-DR and HLA-DM were dispersed, whereas, in PDCs, both HLA-DR and HLA-DM were concentrated in one region, with HLA-DM distributed in close proximity to the cell membrane (Figure 3.15).

(a)



(b)

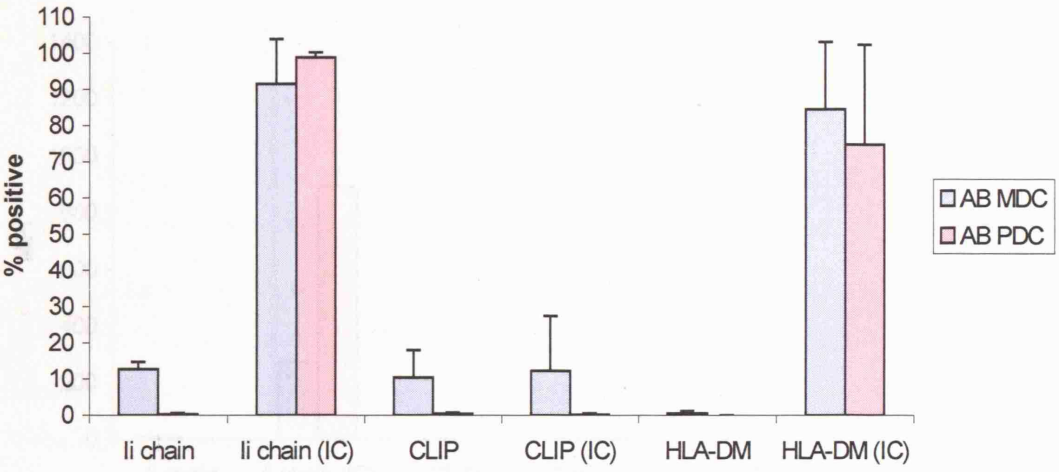
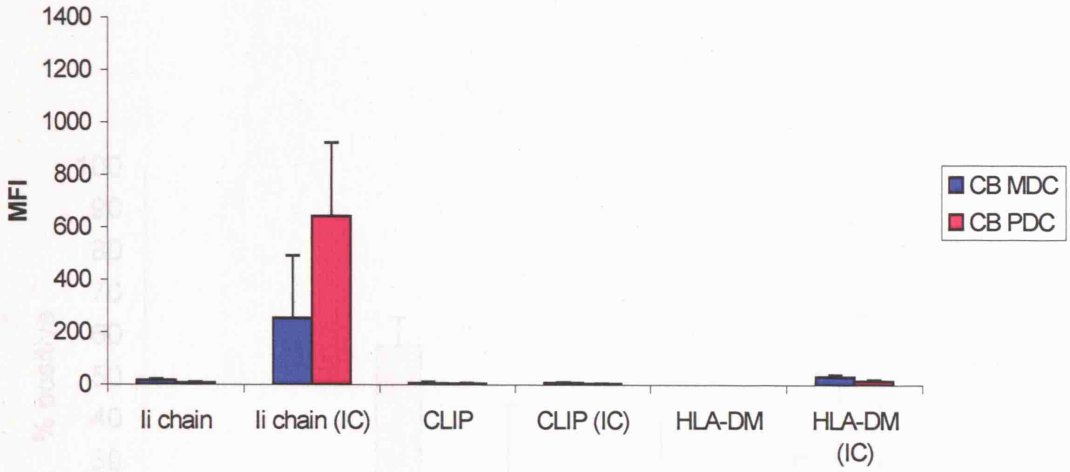


Figure 3.12. Percentage expression of molecules involved in the antigen processing pathway by MDCs and PDCs present in CB and APB.

The expression of the various molecules involved in antigen processing was studied by flow cytometry. Both (a) CB and (b) APB MDCs and PDCs were labelled with MoAbs and the percentage of cells positive for a marker were assessed. The results are shown as mean ± SD of at least three individual experiments. N.B. (IC) symbolises the intracellular staining of markers.

(a)



(b)

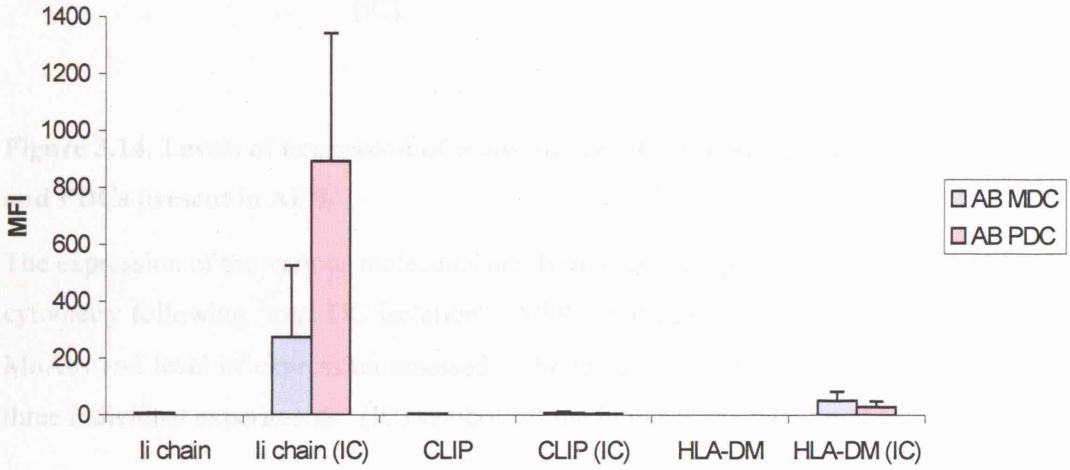


Figure 3.13. Levels of expression of molecules involved in the antigen processing pathway by MDCs and PDCs present in CB and APB.

The expression of the various molecules involved in antigen processing was studied by flow cytometry. Both (a) CB and (b) APB MDCs and PDCs were labelled with MoAbs and level of expression assessed. The results are shown as mean ± SD of at least three individual experiments. (IC) is intracellular staining of markers.

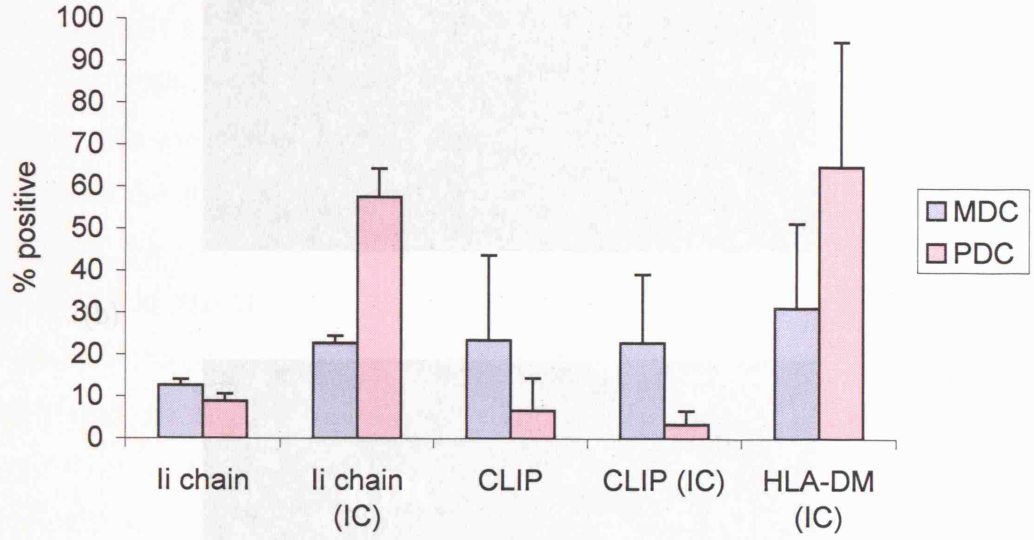
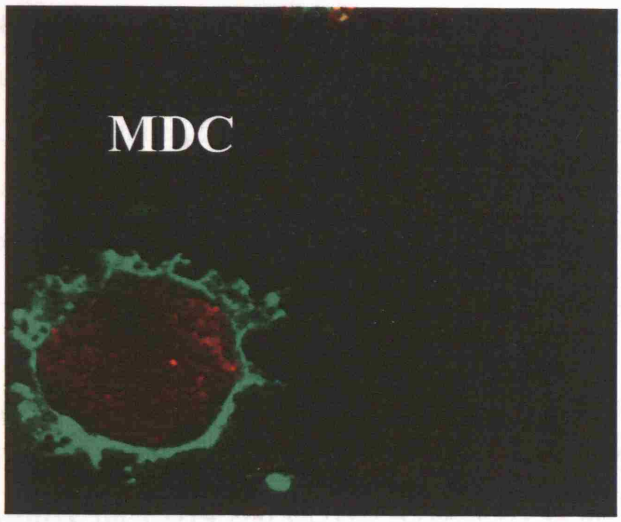


Figure 3.14. Levels of expression of molecules involved in antigen processing by MDCs and PDCs present in APB.

The expression of the various molecules involved in antigen processing was studied by flow cytometry following 'total DC isolation'. MDCs and PDCs from APB were labelled with MoAbs and level of expression assessed. The results are shown as mean ± SD of at least three individual experiments. (IC) symbolises the intracellular staining of markers.

(a)



(b)

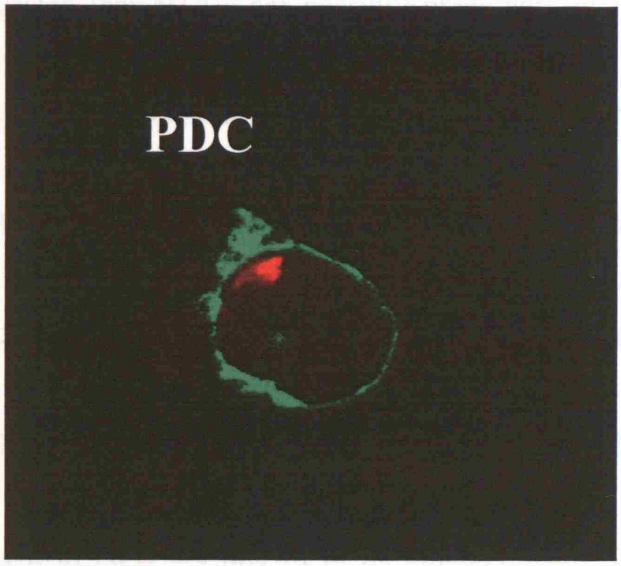


Figure 3.15. Confocal images of dendritic cell subsets.

MDCs and PDCs were isolated by MACS technology and the surface of the cell labelled with HLA-DR, conjugated to FITC, which fluoresced green. Intracellular labelling of HLA-DM with PE fluorophore (red) showed the distribution of HLA-DM molecules within each DC subset. With (a) MDCs, HLA-DM was dispersed randomly in association with the expression of the HLA-DR molecules, whereas, in (b) PDCs, the HLA-DM molecules were concentrated in a single area of the cell together with the surface HLA-DR molecules. The images were produced in collaboration with Prof. Fernandez's laboratory, University of Essex.

3.3 Discussion

As DCs originate from the BM and are distributed to various organs via the blood, precursor/immature DCs are expected to be present in peripheral blood. Cultured cells (e.g. MDDCs) may undergo maturation and may not be representative of blood DCs (Nijman et al., 1995). Blood DCs represent a more physiological cell type that has not been exposed to exogenous cytokines (Hart and Hill., 1997). Therefore, in this thesis DC subsets freshly isolated from CB or APB were used. Initial studies used total DCs, but in subsequent studies, as a result of the availability of DC specific MoAbs, separated DC subsets were used. The results of this thesis reaffirmed the differences between MDCs and MDDCs, since MDCs were negative for CD1a, but positive for CD14 and CD11b (O'Doherty et al., 1994; Thomas and Lipsky, 1994; Zhou and Tedder, 1995; Fearnley et al., 1997; Patterson et al., 2005). It could be suggested that MDCs are possibly precursors of MDDCs (or dermal DCs), since DC precursors migrate from the BM via the blood to the tissues of the body, where they differentiate into dermal DCs and LCs, both expressing HLA class II and CD1a molecules. However, dermal DCs also express CD11b (Patterson et al., 2005). Tissue DCs require appropriate signals from pathogens or inflammatory cytokines to mature and migrate to secondary lymphoid tissues. PDCs differ from the MDCs in that they lack CD11c expression and migrates directly from the BM via the blood to the T cell areas of secondary lymphoid tissues where they secrete high levels of IFNα in response to viruses (Rissoan et al., 1999; Siegal et al., 1999).

DC subsets present in APB are known to be phenotypically distinct due to their surface marker expression. The pattern of surface marker expression differentiates them from other cell types and determines their stage of maturation. Therefore, questions arose as to whether CB DC subsets displayed characteristic features of APB DC subsets representative of a distinct phenotypes prompting studies on the phenotype of DC subsets initially studying absolute cell counts. The study of absolute DC counts showed differences in the ratios of MDC:PDC between CB and APB and revealed that PDCs were the main subset present in CB and MDCs were the quantitatively dominant subset in APB. The results are in agreement with previously established reports on DC subset prevalence (Fearnley et al., 1999; Maraskovsky et al., 2000; Borras et al., 2001; Sorg et al., 1999). Researchers have

also suggested that the number of PDCs in healthy children is age dependent, high at birth (30-40%) and diminishing to adult levels (10-15%) during the first few years of life, whereas MDC counts do not change significantly with age, findings which support the results of this thesis (Teig et al., 2002; Sun et al., 2003; Dakic et al., 2004). However, contradictory studies have reported MDC presence in a slightly higher proportion than PDCs in both CB and APB (Schibler et al., 2002; Hagendorens et al., 2003; Darmochwal-Kolarz et al., 2004). Some of the discrepant results may also be explained by the location of the cells at the time of withdrawal from the donor or by the alternative methodologies used (Szabolcs et al., 2003a).

Absolute counts were higher in CB than APB contradicting reports showing reduced absolute numbers of DCs in neonatal mice compared to adults (Muthukkumar et al., 2000; Dadaglio et al., 2002; Sun et al., 2003; Dakic et al., 2004). The high CB DC counts may be explained by the stress experienced during either vaginal delivery or caesarean section, as it has been shown that physical and surgical stress induces a rise in absolute DC counts (Ho et al., 2001).

The 'whole blood' technique was used for absolute cell counting. The advantage of this method was the inclusion of all cell populations, and that direct sampling of blood DCs could be performed rapidly using small volumes of blood (Mason et al., 2005). In addition, there was no wash step following red cell lysis, hence, no possibility of cell loss. Mason *et al* also demonstrated that cell loss following Ficoll cell separation led to a biased selection of particular DC subsets.

Following absolute cell counting, DCs were isolated from MNCs. Prior to the discovery of DC specific markers, the method used to isolate 'total DCs' depended on a cocktail of antibodies to remove unwanted cell types. This included the antibody to CD11b initially thought to be present only on monocytes, but was subsequently discovered to be expressed by MDCs (O'Keeffe et al., 2003). CD4 was used in the final stage of positive selection of DCs. The results obtained from this chapter showing that PDCs expressed high levels of CD4 compared to MDCs has also been demonstrated by other researchers (Grouard et al., 1997; Sun et al., 2003). Therefore, incubating MNCs with antibodies to both CD11b and CD4 may

have depleted a sizeable number of MDCs from the final product. At present, the isolation of individual DC subsets relies on the specificity of CD1c and CD304 antibodies, diminishing the possibility of loss of cell numbers and inclusion of other unwanted cell populations. This may help to explain the discrepancy between the two methods of separation, 'total DCs' or 'DC subsets', resulting in differences in expression patterns. Therefore, the possible explanation for the contradiction in HLA-DM expression, between the isolation of 'total APB DCs' and separately isolated DC subsets may be the method of isolation prior to the study.

The phenotype of a DC subset may be an important characteristic that distinguishes MDCs from PDCs, and assesses the stage of maturation of DCs. Therefore, immunophenotyping was carried out on freshly isolated CB DC subsets and the results were in accordance with those obtained comparing APB MDCs and PDCs (O'Doherty et al., 1994; Dzionek et al., 2000; Schibler et al., 2002). The results of this chapter showed that, in general, the percentage of expression of several markers was similar between CB and APB DC subsets, but the level of expression was lower in the CB setting, indicating a quantitative difference that may influence the role of CB DCs as APCs suggesting that CB DCs are present in an environment that could diminish Th1 immune responses and promote tolerance or Th2 immunity. This is substantiated by the lower intensity of expression of HLA-DR on CB MDCs and PDCs compared to APB DC subsets. HLA-DR is a pre-requisite for allostimulation and if the levels of expression of HLA-DR are low on DCs, the allostimulatory capacity will be reduced (Kloosterboer et al., 2004). The lower levels of HLA-DR expression on these cells may explain the reduction in the allostimulatory capacity of CB MDCs (see chapter VI). The intensity of expression of HLA class II on CB DCs was diminished compared to APB, but MDCs continued to express significantly higher levels than PDCs. This has been previously demonstrated in APB (Grouard et al., 1997; Robinson et al., 1999). MDCs are more likely to provide a potent signal to the responding T cells by occupying more TCRs due to a higher density of HLA class II molecules on the cell surface. Class II deficient mice are resistant to GvHD, but when class II^- mice are reconstituted with syngeneic class II^+ BM, the mice subsequently present with GvHD (Teshima et al., 2002a). This demonstrates that recognition of HLA class II alloantigen on haematopoietically

derived APCs alone, is sufficient to activate donor $CD4^+$ T cells and cause lethal GvHD. Therefore, the reduced level of expression of HLA class II molecules by CB DC subsets would either activate less T cells and/or tolerise them and this may contribute to a reduction in GvHD. Another interesting finding was the reduced percentage of CB and APB PDCs expressing HLA-DQ and HLA-DP compared to HLA-DR, and the lower density of HLA-DQ on DCs has been demonstrated (Knight et al., 1987). Therefore, HLA-DQ and HLA-DP could be used as markers to differentiate between MDCs and PDCs.

Other molecules selected for DC subset identification, also showed differences between CB and APB DCs and included CD123 and the CD45 isoforms (CD45RA and CD45RO) (O'Doherty et al., 1993; O'Doherty et al., 1994). The level of expression of CD123 (IL-3R) on CB DC subsets was enhanced compared to APB, alluding to the concept of an increased need for this receptor in the CB setting, suggesting CB DCs may be more susceptible to environmental factors and therefore, require survival signals such as IL-3, a cytokine for PDC survival (Grouard et al., 1997). The results from this chapter showed that the majority of CB MDCs and both CB and APB PDCs were highly positive for CD45RA suggesting an immature phenotype compared to APB MDCs, since CD45RA is a marker of T cell naivety (Young et al., 1997). The immature status of CB DCs was further supported by the markedly reduced expression of CD45RO, a memory marker, on CB MDCs compared to APB MDCs, and lack of expression on PDCs in general.

A molecule that has not been previously found on freshly isolated CB DCs is HLA-G. MoAbs to only two isoforms of HLA-G (G1 and G5) were available for flow cytometric analysis. Therefore, the presence of the other isoforms (G2, G3, G4 and G5) was assessed by RT-PCR. HLA-G1, and its corresponding soluble isoform, HLA-G5, were present in CB MDCs to a greater extent than in APB DCs. These isoforms appear to be the main functional forms on the CB DC surface and intracellularly due to the high intensity of the bands on the gel (Figure 3.7) compared to the positive control band (JEG-3). Both HLA-GI and G5 are important in immunosuppressive effects (Bainbridge et al., 2000a; Le Bouteiller and Solier, 2001). The function of membrane HLA-G1 has been described but experiments

have also shown secretion of soluble HLA-G5 protein by allospecific $CD4^+$ T cells, which suppressed alloproliferative T cell responses (Lila et al., 2001). Experiments to block HLA-G expression would help in determining the role of HLA-G in CB DCs. The shorter HLA-G isoforms (HLA-G2, -G3, and -G4) produced intracellularly, are sequestered in the ER and so do not reach the cell surface (Bainbridge et al., 2000b; Mallet et al., 2000) and may function only to support HLA-E expression and modulate CD94/NKG2 ligand interaction. This function may be attributed to PDCs in which HLA-G3 mRNA was detected. The data indicates that CB DCs can potentially express HLA-G to a greater extent than that described for APB.

Another interesting observation was the presence of the early HSC markers, CD133 and CD34, on CB MDCs and PDCs but not on APB DC subsets. The presence of CD34, CD45A, CD133 and HLA-G would reinforce the suggested immature/precursor status of CB DC subsets.

Other molecules involved in strengthening the interactions between a DC and T cell during antigen presentation include adhesion and costimulatory molecules, which are expressed on DCs. Following the initial contact between the MHC-peptide complex on DCs and the TCR on T cells (signal 1), these molecules subsequently engage receptors on T cells and provide signal 2 that influences T cell activation, expansion and phenotype. The delivery of signal 1 without signal 2 can result in tolerance induction (Schwartz, 1990; Kundig et al., 1996). When the DC-T cell interaction is altered, there is a marked reduction in the T cell proliferative response initiated by DCs (Bachmann et al., 1997; Masten et al., 1997). Cosignalling pathways such as CD80-CD86/CD28, CD40/CD40L or CD11a/ICAM-1 are critical in the development of immune responses and blocking these pathways has improved transplant outcomes resulting in transplantation tolerance and T_{reg} production (Lenschow et al., 1996; Isobe et al., 1997; Koenen and Joosten, 2000; Alegre et al., 2001). The lack of costimulatory signals leads to anergy where there is an unresponsive state of T cells to antigen. Therefore, the results of this thesis showing a reduced percentage of CD86 molecules and a reduced level of expression of CD11a on CB PDCs compared to CB MDCs, could suggest delayed or weak DC-T

cell interactions resulting in tolerance induction, hence, reducing the severity of GvHD following CBT.

The expression of CD86, and CD80 and CD83, on DCs would also indicate DC activation and/or maturation, respectively. Differential expression of the costimulatory molecule, CD86, was evident between MDCs and PDCs, although, there were minimal differences in the results comparing CB and APB. MDCs expressed significantly higher amounts of CD86 than PDCs, suggesting an activated state of MDCs, but not necessarily maturity as MDCs were negative for CD80 and CD83 molecules. The low level of expression of CD86 on PDCs and lack of CD80 expression further confirms the immaturity of these cells and their differential status compared to MDCs.

The study of the molecules Ii chain, CLIP and HLA-DM, involved in the HLA class II antigen processing pathway revealed differential expression by CB MDCs and PDCs, which could reflect specialised functions or different states of activation for each DC subset. Surface Ii chain was expressed on a significantly higher percentage of CB MDCs than CB PDCs. Although, the percentage of intracellular Ii chain was similar between CB MDCs and PDCs, the level of expression was significantly higher in CB PDCs. This pattern of expression was repeated with APB DC subsets. Therefore, the differences between MDCs and PDCs may be due to the Ii chain, as different variants of Ii chain are targeted to either the endosomes (p35) or the cell surface (p33) (Anderson and Roche, 1998), suggesting the Ii chain of MDCs may be targeted to the cell surface and that of PDCs to the endosomes. Newly synthesised HLA class II molecules first appear at the cell surface as complexes with Ii chain (Saudrais et al., 1998) before entering the endosomal compartment and then internalise for Ii chain degradation by cathepsin S and peptide loading (Maurer et al., 1998). CLIP expression was present in MDCs but absent in PDCs suggesting that the Ii chain within an MDC may be degraded as it is sensitive to protease digestion (Park et al., 1995), but Ii chain is not readily degraded in PDCs. Cathepsin S is an enzyme required for Ii chain degradation and mice lacking this enzyme have demonstrated inhibition of Ii chain degradation (Nakagawa et al., 1999), which may be a characteristic of PDCs. The deficit of cathepsin S in PDCs may also be

supported by the results showing equivalent expression of intracellular HLA-DM in CB and APB DC subsets. HLA-DM catalyses the release of CLIP, but CLIP is not present in PDCs, and Ii chain is abundant in the PDC subset, suggesting the process of Ii chain degradation is defective or delayed due to the lack of cathepsin S. Therefore, studying the expression of this enzyme within the DC subsets may help to explain the differences in CLIP and Ii chain expression.

The general findings showed that there was an overall reduced level of expression of the majority of markers studied on CB DC subsets, in particular, MHC molecules, important in antigen processing and presentation, and the preferential expression of markers of immaturity. This is where the phenotypic difference between CB and APB DC subsets seems to lie.

Chapter IV

RESULTS 2: THE CYTOKINE PROFILE OF DENDRITIC CELL SUBSETS

4.1 Introduction

Dendritic cells must be stimulated to act as nature's adjuvants for the induction of immunity and can be triggered by exposure to bacterial (LPS, CpG DNA motifs) or viral (dsRNA) components, as well as T cell interactions (CD40 ligation) (Steinman et al., 2000). Upon stimulation, DCs upregulate surface molecules and begin to secrete cytokines which exert an autocrine effect on DCs and influence Th differentiation. The cytokine profile varies according to the DC subset studied (Almeida et al., 1999) and the predominant cytokine produced by stimulated MDCs is IL-12, and IFNα is secreted upon PDC stimulation (Siegal et al., 1999). Since DC subsets secrete differential cytokines upon maturation, it was of interest to study the cytokine profile of CB MDCs and PDCs to determine if CB DCs secreted equivalent cytokines or had a disparate cytokine profile compared to APB DCs, which may influence the function of CB DCs.

Cytokine secretion results from DC recognition of conserved molecular patterns of microbial components via TLRs. TLRs signal via pathways that activate various transcription factors, triggering cytokine production. This is reproduced *in vitro* by exposure to microbial products such as LPS (Sallusto and Lanzavecchia, 1994), inducing DCs to produce varying amounts of immune enhancing cytokines. Therefore, the exact cytokine repertoire expressed will depend on the nature of the stimulus (LPS or CpG) and the existing cytokine microenvironment (CB or APB), as well as the maturation stage of the DC subsets (immature versus mature). Therefore, the cytokine profile of steady state and stimulated DC subsets was determined by incubating MDCs with IL-4 and PDCs with IL-3, or stimulating MDCs with LPS and PDCs with CpG, respectively (Jarrossay et al., 2001; Kadowaki et al., 2001b; Kadowaki et al., 2001a; Krug et al., 2001b; Hornung et al., 2002). Time course experiments were carried out (12, 18 and 24 hr) for up to 24 hr and the supernatants were removed to assess cytokine secretion.

As stimulated DCs are known to secrete a variety of cytokines, the following cytokines were studied: IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-13, GM-CSF, IFNα, IFNγ and TNFα. In order to analyse the cytokine profile of CB DC subsets, a novel multiple cytokine detection system using Luminex® Technology was

applied. Using this technique it was possible to study multiple cytokines secreted simultaneously by CB DC subsets prior to and following activation with specific stimuli.

4.2 Results

4.2.1 The kinetics of the cytokine profile

The kinetics of the cytokines produced at steady state (Appendix 3) or following stimulation (Figures 4.1 to 4.10) were studied. All results are shown as mean ± SD of three independent experiments. The lower limit of cytokine detection was 6.9 pg/ml, except for IFNγ which was 12.3 pg/ml. The upper limit of detection was 5000 pg/ml for all cytokines studied and any values above this were denoted ≥ 5000 pg/ml. The trend between individuals was similar but there were different levels of secretion. This variation in secretion was detected for different cytokines produced by a single individual, i.e. if high levels of a cytokine were detected for one individual, this was observed for all other cytokines produced by that individual. The cytokines absent from both CB and APB DC subsets were IL-4 and IFNγ.

Steady state CB MDCs and PDCs, culture with IL-3 and IL-4, respectively, produced low concentrations of proinflammatory cytokines. The results were equivalent in the adult setting, with low or no production of the various cytokines studied (see Appendix 3 for kinetic studies on steady state DCs).

4.2.2 Proinflammatory cytokines

IL-1β, IL-6 and TNFα were studied and are known to be proinflammatory cytokines produced following encounter with pathogens.

4.2.2.1 Interleukin-1 beta

IL-1β was produced following stimulation of both CB MDCs and PDCs (Figure 4.1). CB MDCs stimulated with LPS induced an initial burst of production of IL-1β but after 12 hr levels remained constant. CB PDCs stimulated with CpG produced detectable levels of IL-1β, which was consistent over 24 hr (Figure 4.1a). There was

a steady increase in IL-1β production over the 24 hr time period. The pattern was repeated for different samples, however, the levels of secretion varied. APB MDCs had a slower kinetic profile compared to CB MDCs, but increased to levels similar to CB by 24 hr. MDCs secreted higher amounts of IL-1β than PDCs.

4.2.2.2 Interleukin-6

Stimulation of CB MDCs and CB PDCs with LPS and CpG, respectively, produced IL-6. There was a significant production of IL-6 by CB MDCs (≥ 5000 pg/ml) (Figure 4.2a), but CB PDCs were overall less effective secretors of IL-6 (Figure 4.2b). APB MDCs secreted similar levels of IL-6 to CB MDCs, but APB PDCs did not secrete detectable IL-6 following treatment (< 6.9 pg/ml) (Figure 4.2c and 4.2d). Overall, MDCs produced higher concentrations of IL-6 than PDCs.

4.2.2.3 Tumour Necrosis Factor-alpha (TNFα)

LPS-stimulated CB MDCs secreted significantly higher concentrations of TNFα compared to CpG-stimulated CB PDCs (Figure 4.3a and 4.3b) ($p < 0.05$). The limit of production for CB MDCs was established at the 18-hr period of culture (Figure 4.3a). CpG-stimulated CB PDCs secreted increasing amounts of TNFα over 24 hr (Figure 4.3b). APB MDCs stimulated with LPS secreted higher levels than CB and did not reach a peak in production (Figure 4.3c). APB PDCs did not produce detectable levels of TNFα (Figure 4.3d).

4.2.3 Secretion of Interleukin-2

CB PDCs during culture with IL-3 (Appendix 3) or CpG (Figure 4.4b) solely produced IL-2. If there was any secretion by MDCs or APB PDCs, it was below the level of detection (< 6.9 pg/ml).

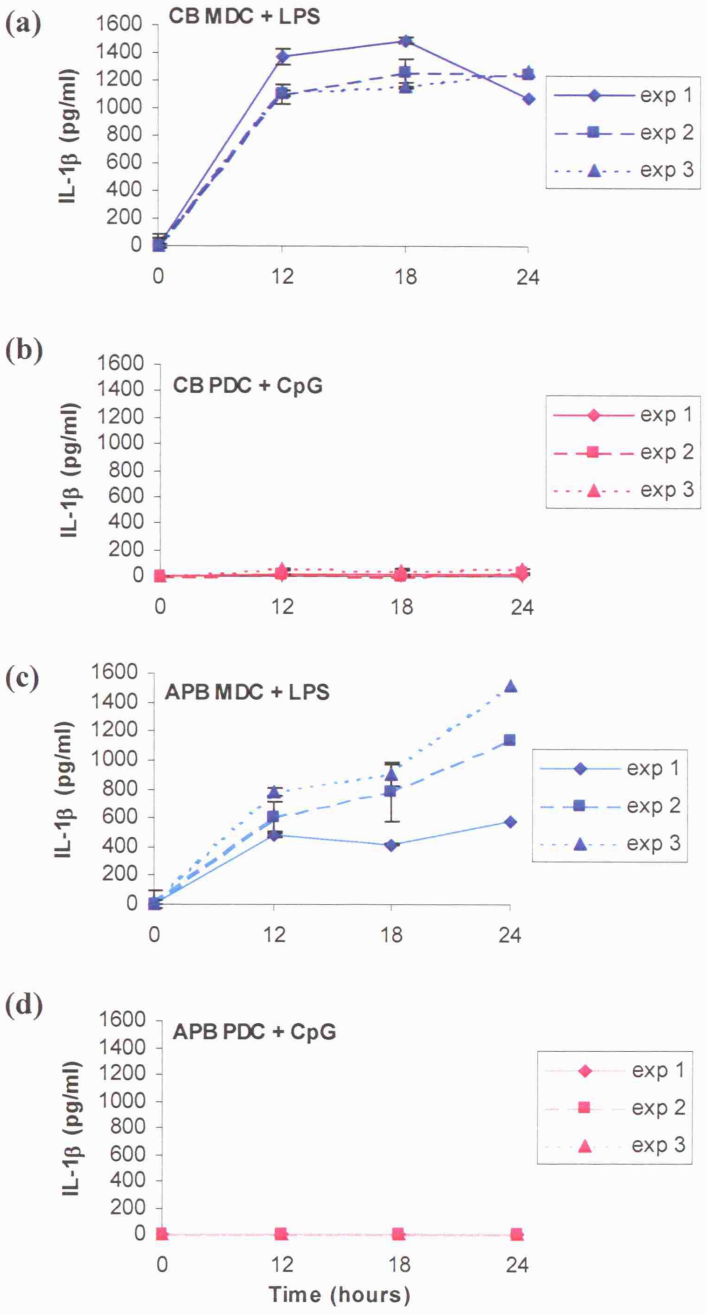


Figure 4.1. Secretion of IL-1β following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-1β detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

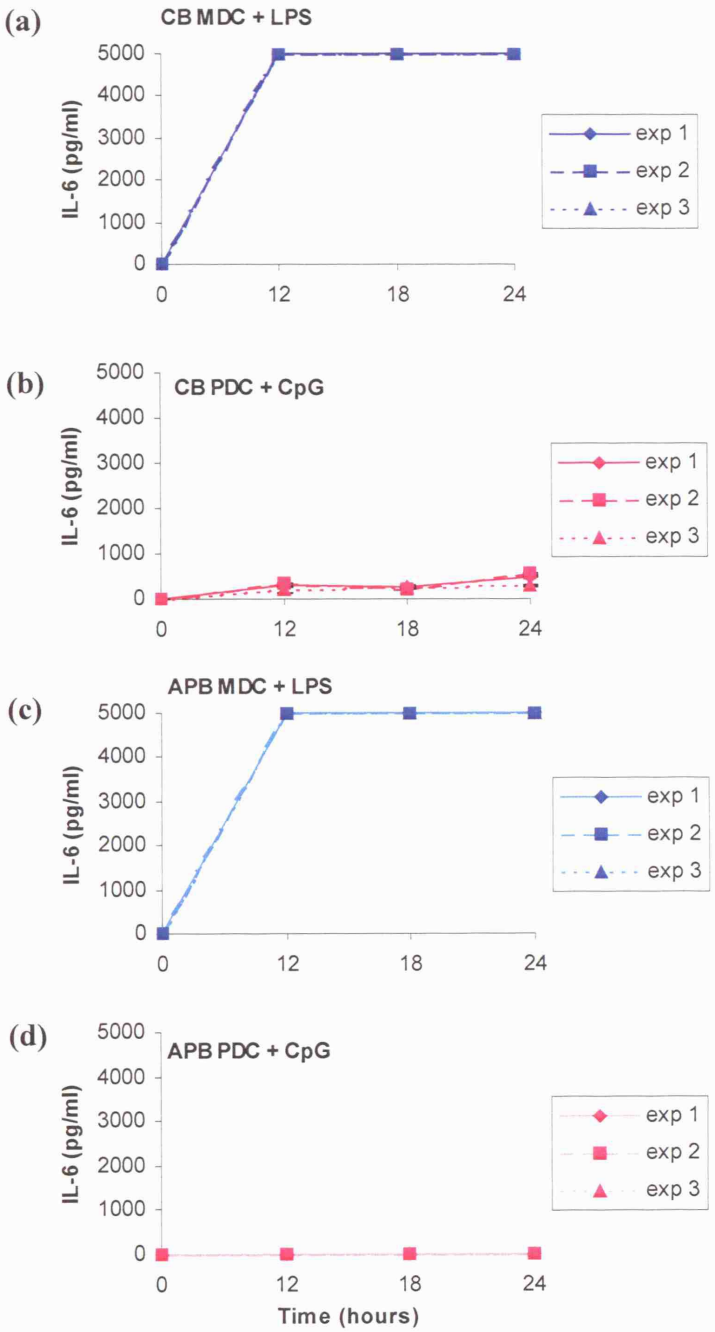


Figure 4.2. Secretion of IL-6 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-6 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

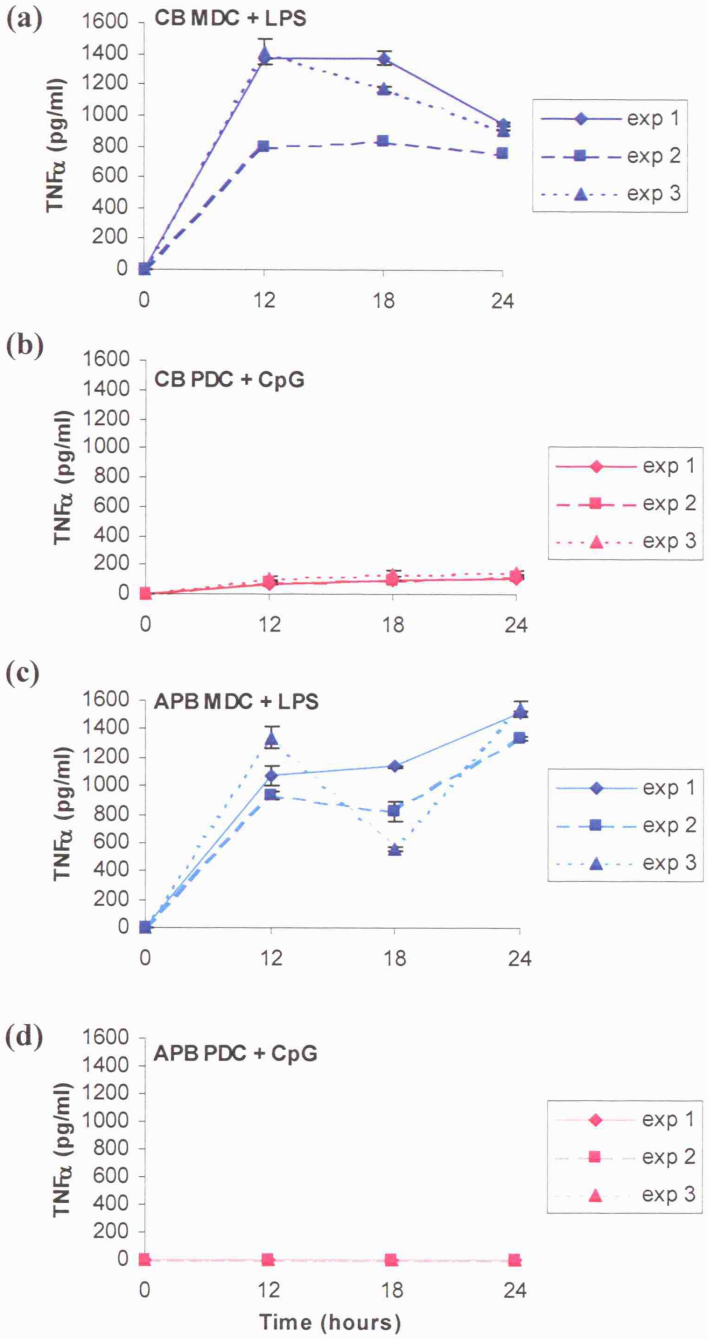


Figure 4.3. Secretion of TNFα following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for TNFα detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

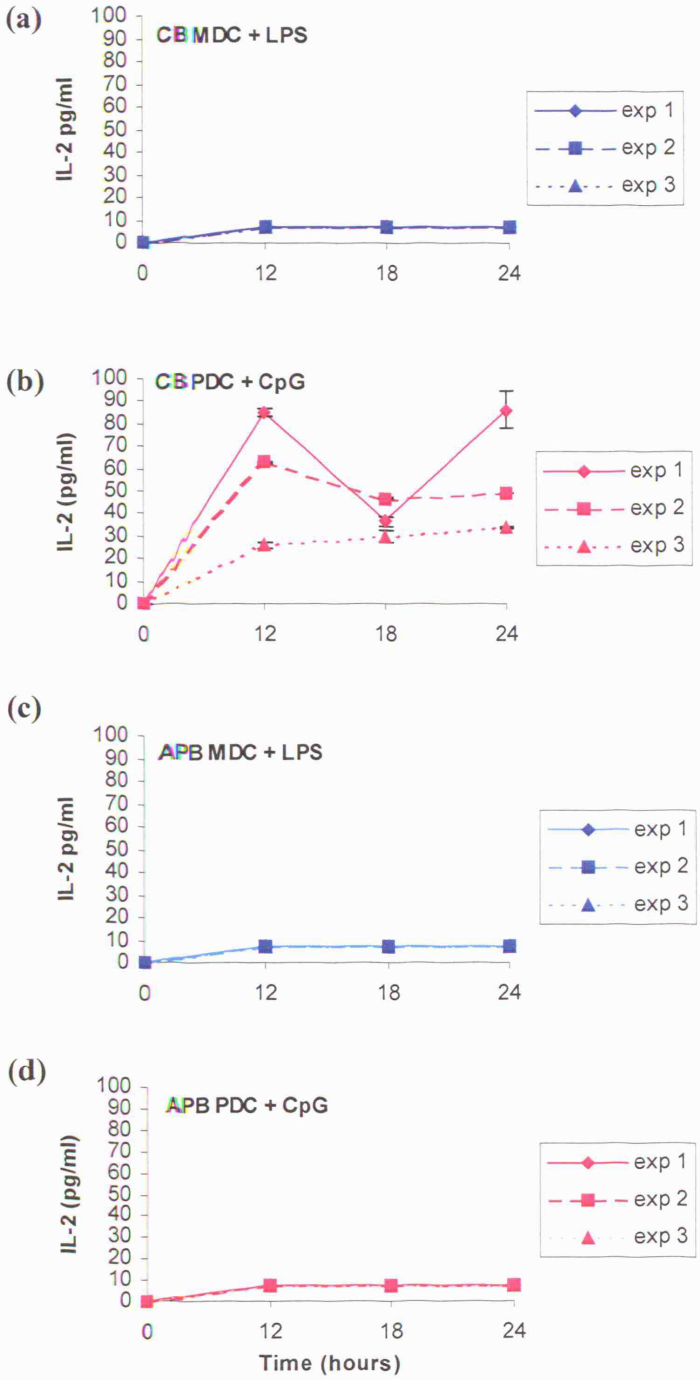


Figure 4.4. Secretion of IL-2 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-2 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

4.2.4 Secretion of Granulocyte Monocyte-Colony StimulatingFactor

CB MDCs secreted significantly more GM-CSF over 24 hr than CB PDCs ($p < 0.05$), but the trend was similar, steadily increasing throughout the culture period (Figure 4.5a and 4.5b). APB MDCs produced GM-CSF, but secretion between adult individuals varied considerably (Figure 4.5c). APB PDCs did not produce detectable levels of GM-CSF (Figure 4.5d).

4.2.5 Secretion of Dendritic cell subset specific cytokines

The two main cytokines that differentiate MDCs from PDCs are IL-12 secreted by MDCs and IFNα produced by the PDC subpopulation. Therefore, these cytokines were also investigated (Figure 4.6 and 4.7).

4.2.5.1 Interleukin-12

Stimulation of CB and APB MDCs with LPS was sufficient to produce low amounts of IL-12 (Figure 4.6a and 4.6c). The levels for CB peaked at 18 hr after which time they decreased. In the adult setting, secretion of IL-12 remained constant throughout the culture period. There was also a variation in the levels of IL-12 secretion between individuals. There was no detectable IL-12 secretion following stimulation of CB or APB PDCs (Figure 4.6b and 4.6d).

4.2.5.2 Interferon-alpha (IFNα)

CB MDCs secreted higher concentrations of IFNα compared to CB PDCs (Figure 4.7a and 4.7b). APB MDCs did not secrete detectable concentrations of IFNα (Figure 4.7c). APB PDCs cultured with IL-3 secreted low amounts of IFNα, (Appendix 3), but levels were increased 8-fold following stimulation with CpG (Figure 4.7d) and compared to CB PDCs. However, secretion of IFNα by APB PDCs was highly variable between individual samples.

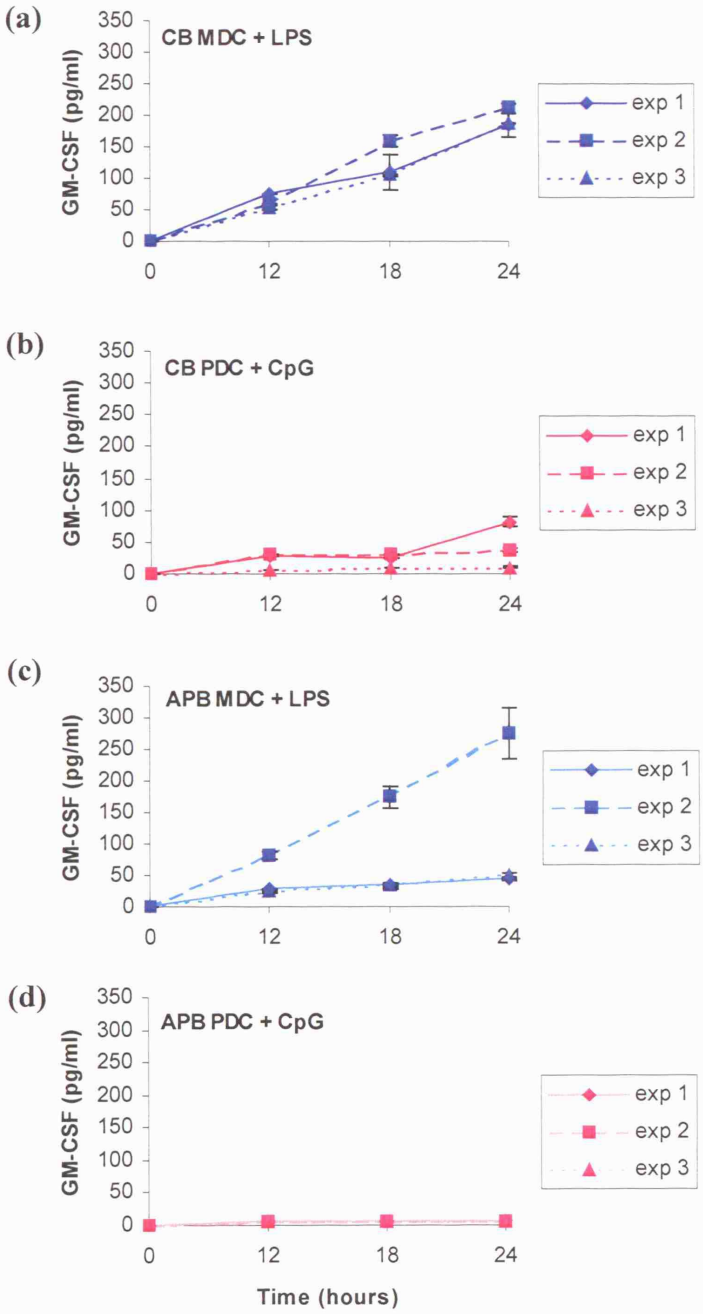


Figure 4.5. Secretion of GM-CSF following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for GM-CSF detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

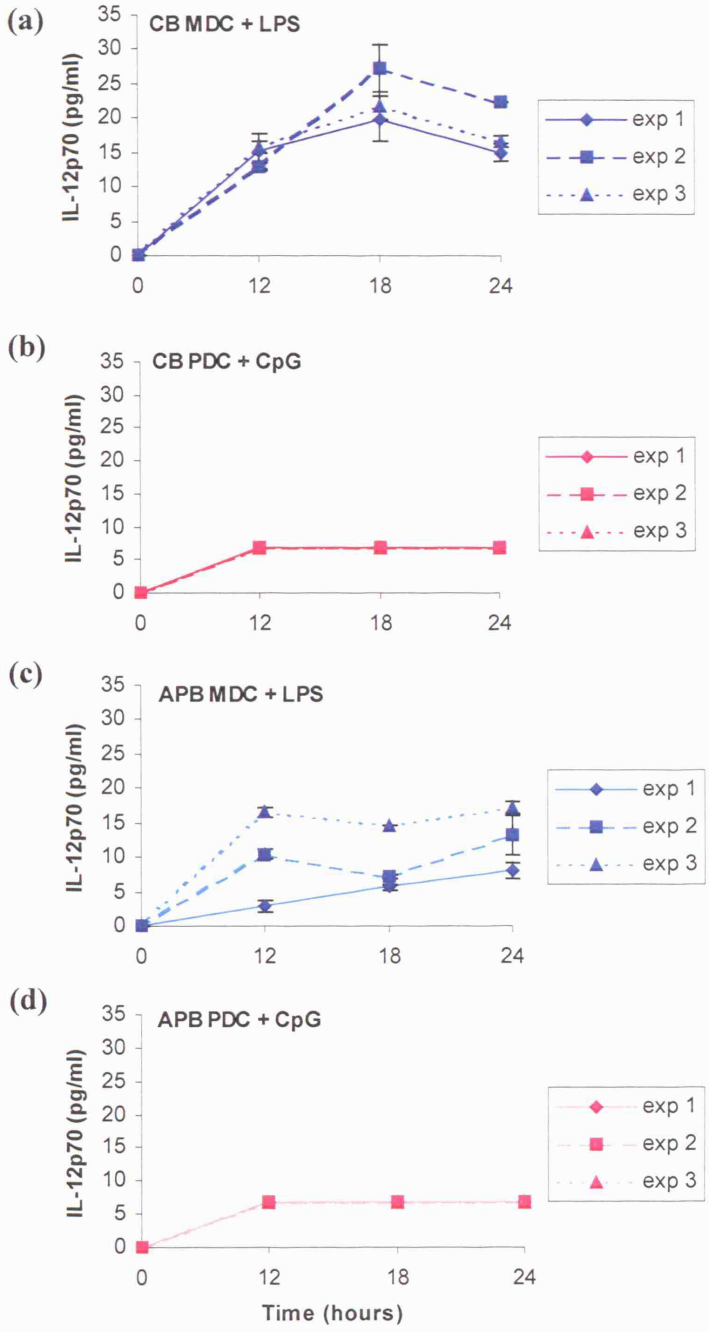


Figure 4.6. Secretion of IL-12 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-12 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

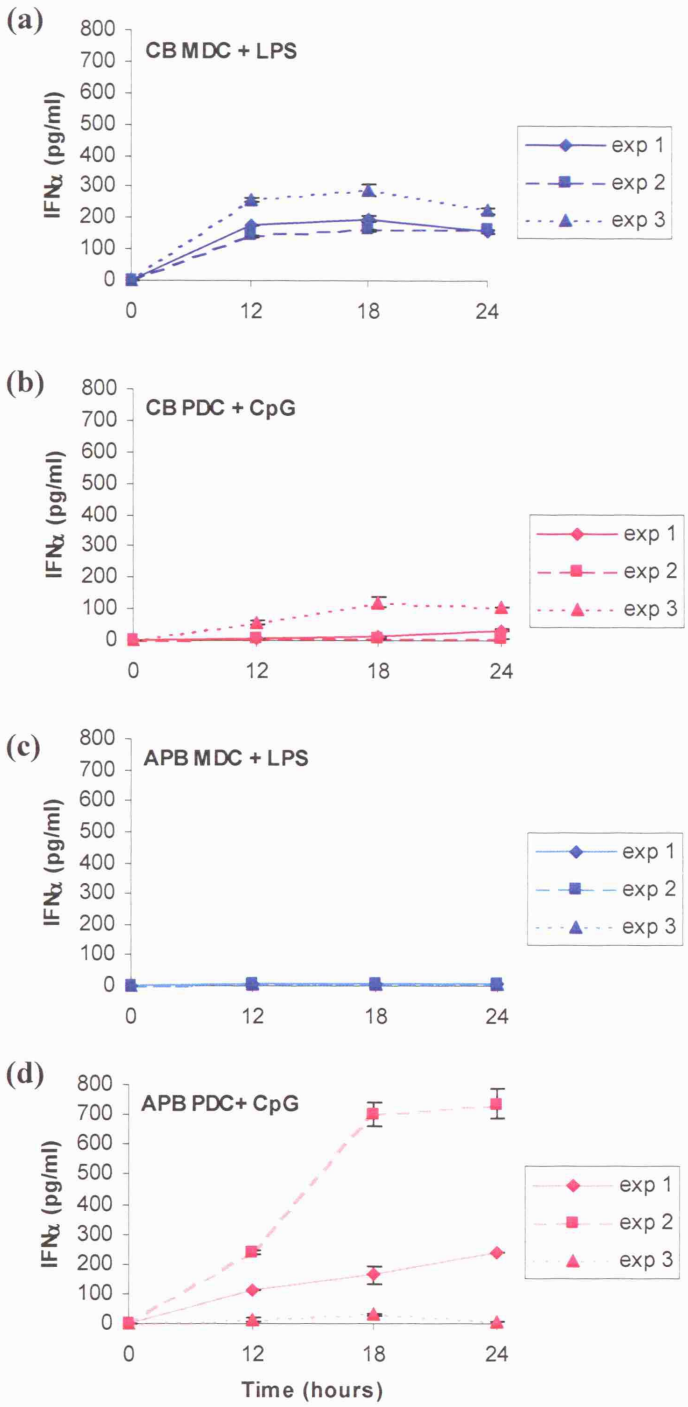


Figure 4.7. Secretion of IFNα following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IFNα detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

4.2.6 Secretion of Th2 cytokines

The various Th2 cytokines secreted by a DC subset included IL-10 and IL-13, but IL-4 production was not detectable (Figure 4.8 and 4.9).

4.2.6.1 Interleukin-10

Production of IL-10 was restricted to LPS-stimulated MDCs, from both CB and APB (Figure 4.8a and 4.8c). The trend for both CB and APB MDCs was similar but there was a difference in the relative levels of production, with APB MDCs producing higher amounts of IL-10 compared to CB MDCs. There was also greater variability between different APB samples (Figure 4.8c). PDCs did not secrete detectable levels of IL-10 when isolated from either CB or APB (Figure 4.8b and 4.8d).

4.2.6.2 Interleukin-13

CB PDCs secreted detectable levels of the IL-13 but CB MDCs did not produce this cytokine (Figure 4.9a and 4.9b). IL-13 production by APB MDCs and PDCs was not observed above the lower limit of detection of 6.9 pg/ml (Figure 4.9c and 4.9d).

4.2.7 Secretion of Interleukin-8

MDCs and PDCs present in both CB and APB secreted IL-8. Both CB MDCs and CB PDCs (Figure 4.10a and 4.10b) produced significantly high amounts of IL-8. APB MDCs produced amounts of IL-8 equivalent to CB DCs, but significantly higher than APB PDCs (Figure 4.10c and 4.10d). The production of IL-8 secretion did not vary considerably after the 12 hr time point.

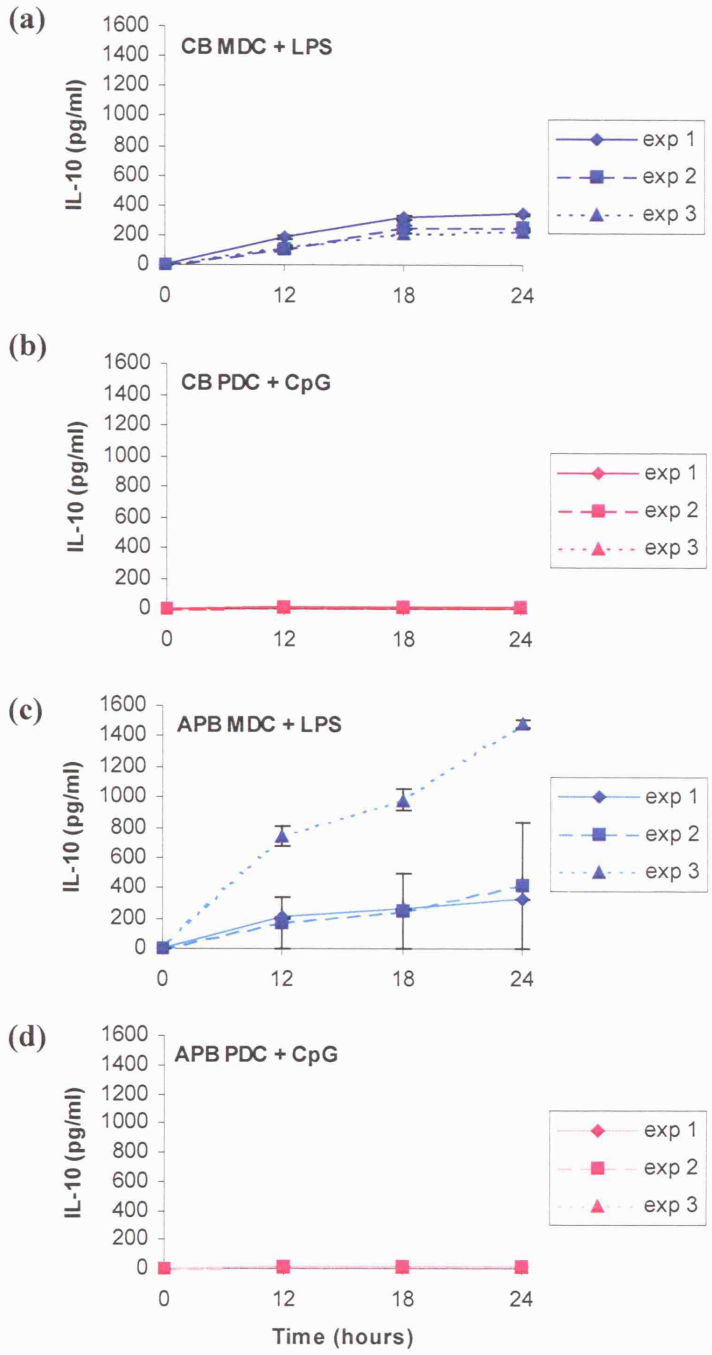


Figure 4.8. Secretion of IL-10 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-10 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

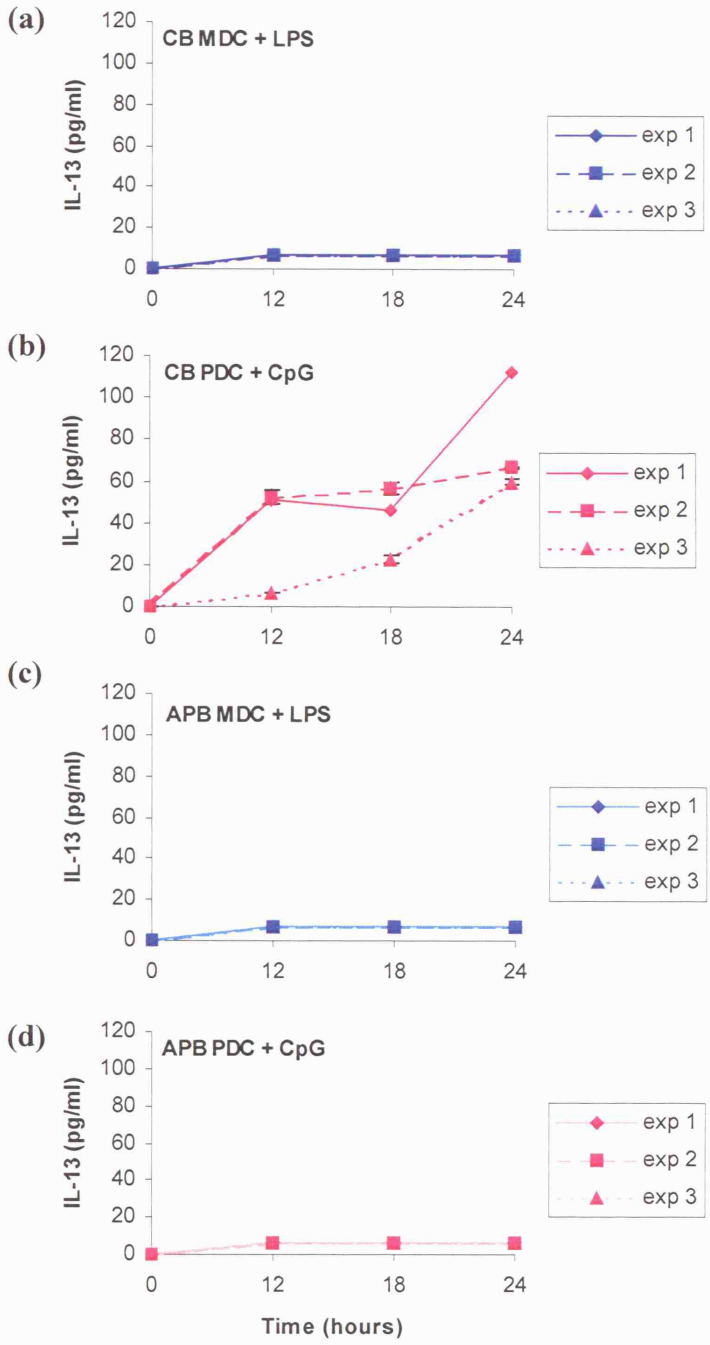


Figure 4.9. Secretion of IL-13 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-13 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

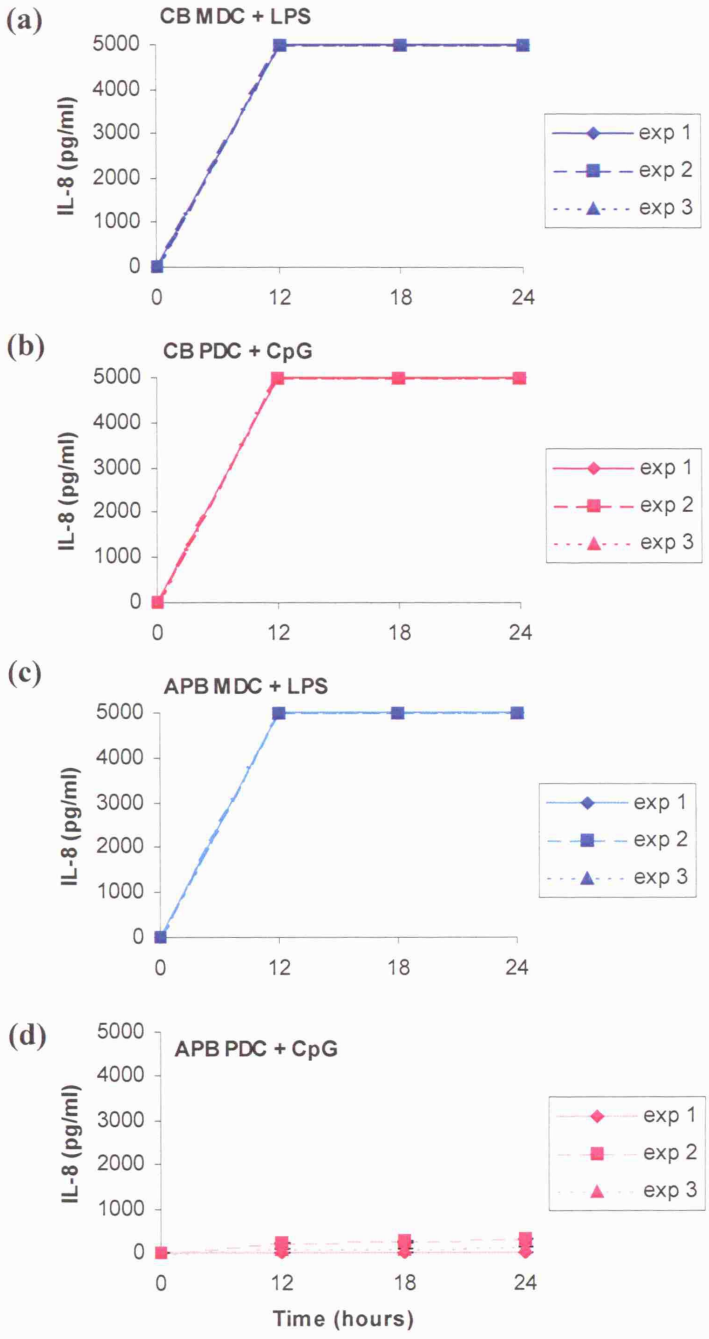


Figure 4.10. Secretion of IL-8 following stimulation of MDCs and PDCs present in CB and APB.

MDCs and PDCs were isolated using the MACS technique and stimulated with LPS and CpG, respectively, over a 24 hr time period before removing supernatants for IL-8 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + LPS (b) CB PDCs + CpG (c) APB MDCs + LPS and (d) APB PDCs + CpG. The results represent three independent experiments.

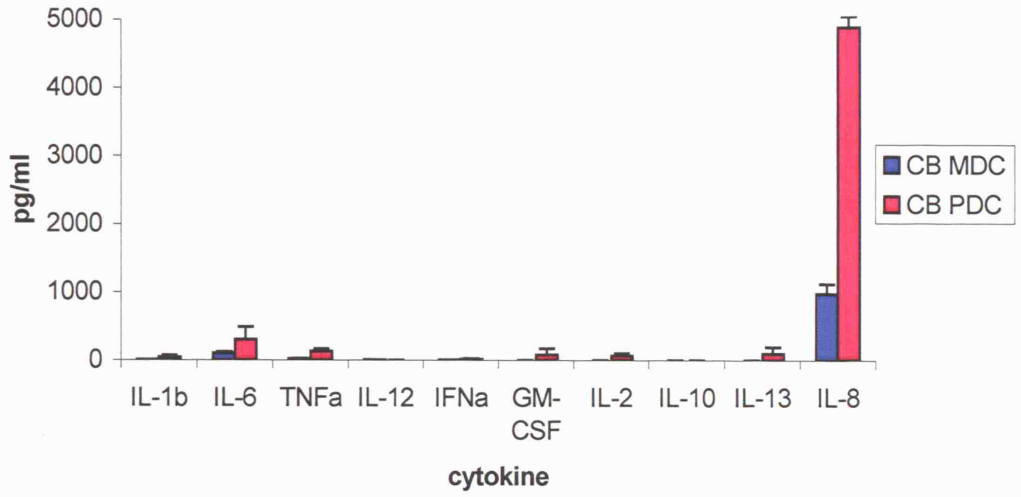
4.2.8 Cytokine secretion following a 24 hour stimulation

The figures 4.11 to 4.14 provide a summary of the cytokines secreted by CB and APB DC subsets following a 24 hr period of co-culture with IL-3 or IL-4 (Figures 4.11 and 4.12) or stimulation with LPS or CpG (Figures 4.13 and 4.14). There was a 10-fold difference between steady state cytokine profile and that following stimulation.

Overall, steady state CB DC subsets secreted higher amounts of more of a variety of cytokines compared to APB DC subsets following culture with IL-3 and IL-4. This was particularly prominent for CB PDCs (Figures 4.11 and 4.12).

Stimulated CB MDCs and PDCs produced similar cytokines, but the concentrations secreted were higher in the MDC subpopulation (Figure 4.13). APB MDCs produced the majority of cytokine studied, but the cytokine profile of APB PDCs was restricted to IL-8 and IFNα secretion (Figure 4.13). MDCs from both CB and APB produced similar levels of the various cytokines studied (Figure 4.14). CB PDCs, however, were more efficient secretors of cytokines compared to their adult counterparts.

(a)



(b)

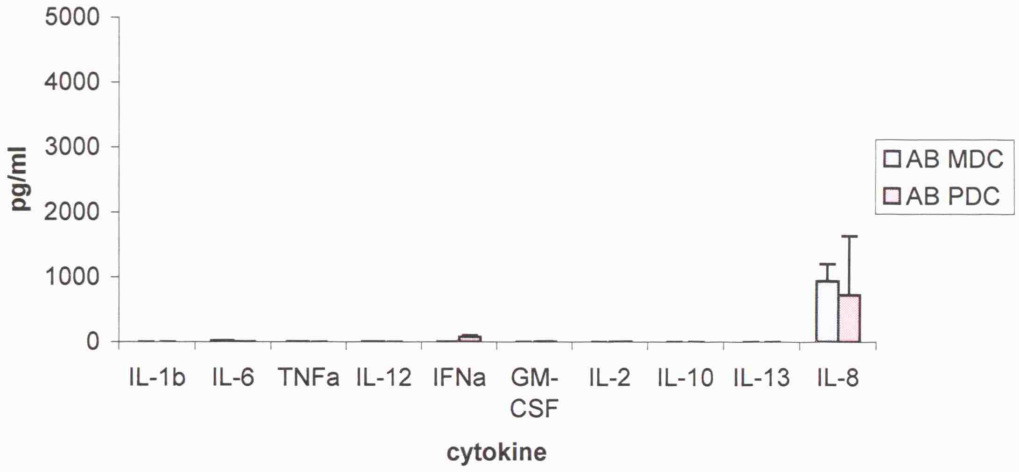
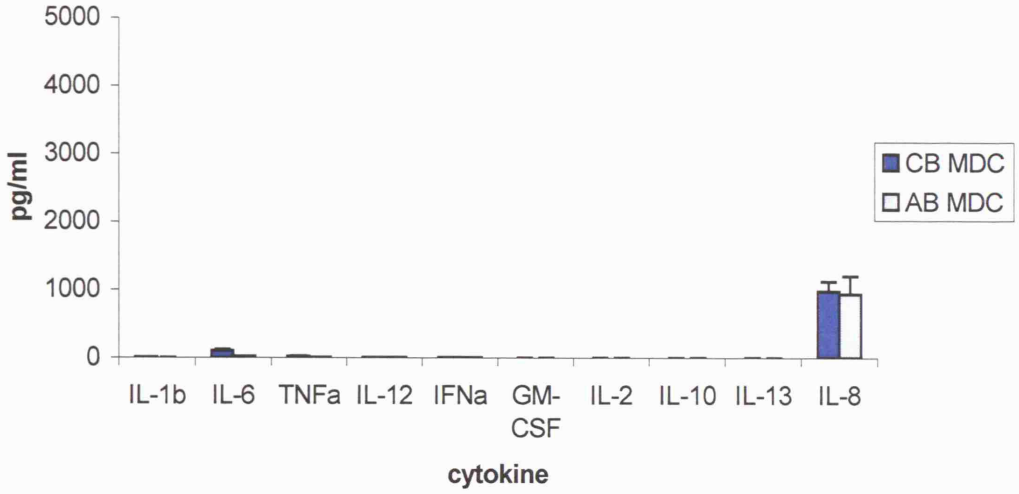


Figure 4.11. A comparison of the cytokines secreted at steady state by MDCs and PDCs isolated from CB and APB.

These graphs summarise the various cytokines studied following a 24-hr culture of DC subsets with IL-4 (MDCs) or IL-3 (PDCs). The cytokines include pro-inflammatory, Th1 and Th2 cytokines, and the chemokine IL-8. (a) compares CB MDCs and PDCs, and (b) compares APB MDCs and PDCs (n = 3).

(a)



(b)

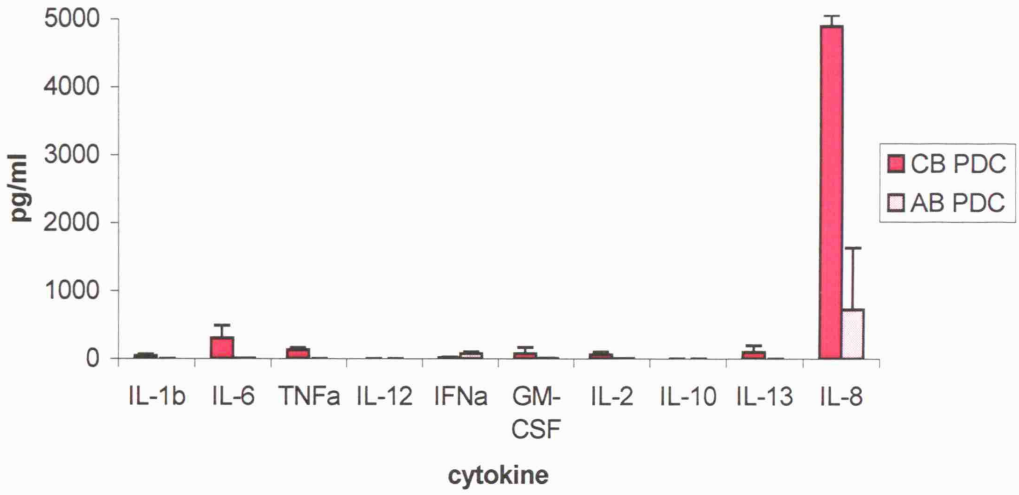
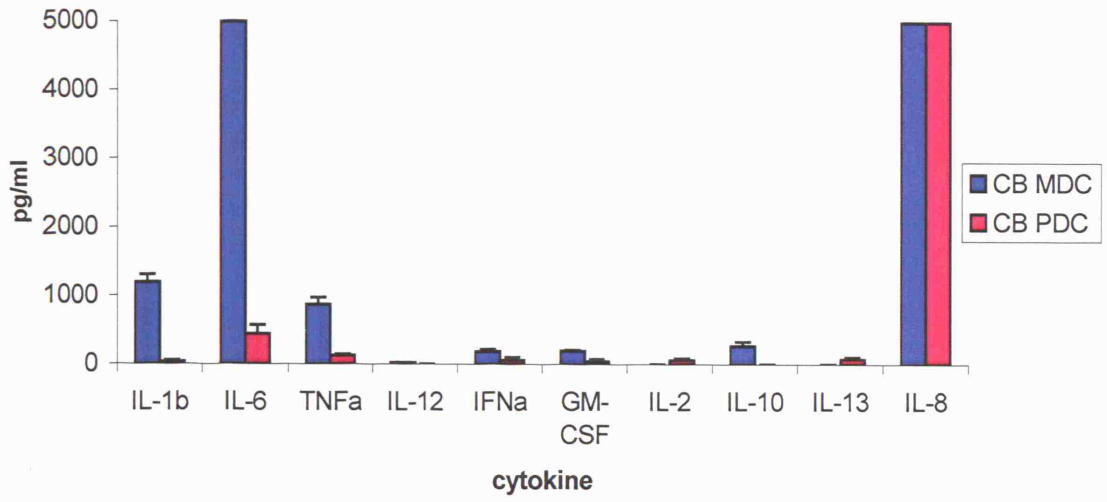


Figure 4.12. A comparison of the cytokines secreted at steady state by MDCs and PDCs isolated from CB and APB.

These graphs summarise the various cytokines studied following a 24-hr culture of DC subsets with IL-4 (MDCs) or IL-3 (PDCs). The cytokines include pro-inflammatory, Th1 and Th2 cytokines, and the chemokine IL-8. (a) compares CB and APB MDCs, and (b) compares CB and APB PDCs (n = 3).

(a)



(b)

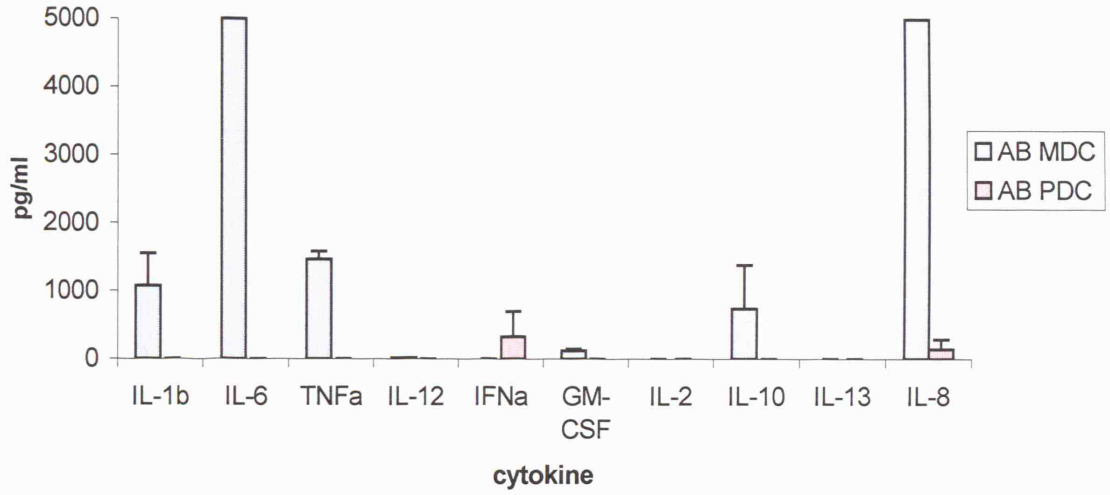
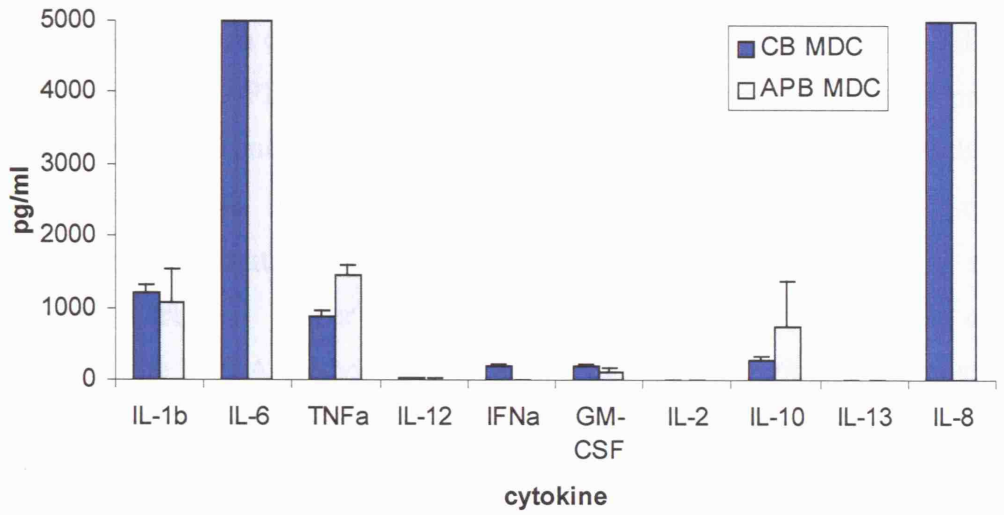


Figure 4.13. A comparison of the cytokines secreted by stimulated MDCs and PDCs isolated from CB and APB.

These graphs summarise the various cytokines studied following a 24-hr stimulation of DC subsets with LPS (MDCs) or CpG (PDCs). The cytokines include pro-inflammatory, Th1 and Th2 cytokines, and the chemokine IL-8. (a) compares CB and APB MDCs, and (b) compares CB and APB PDCs (n = 3).

(a)



(b)

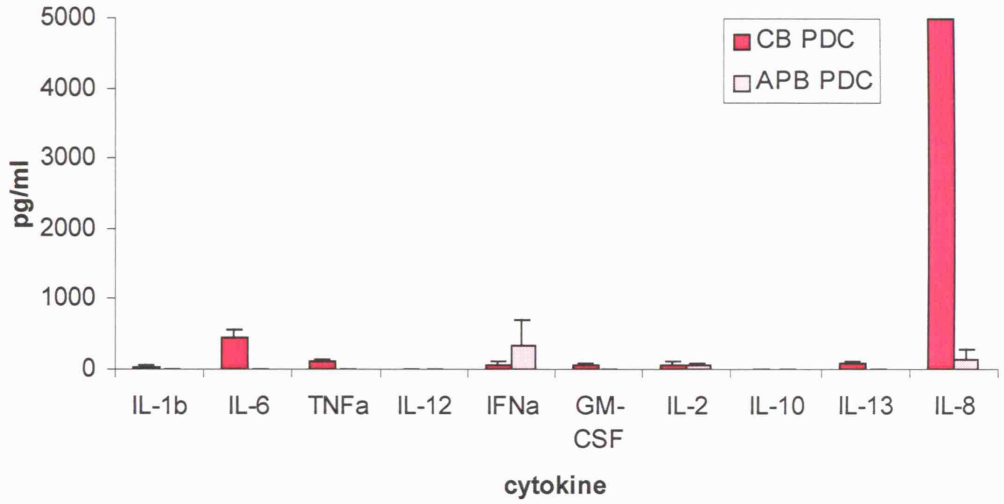


Figure 4.14. A comparison of the cytokines secreted by stimulated MDCs and PDCs isolated from CB and APB.

These graphs summarise the various cytokines studied following a 24-hr stimulation of DC subsets with LPS (MDCs) or CpG (PDCs). The cytokines include pro-inflammatory, Th1 and Th2 cytokines, and the chemokine IL-8. (a) compares CB and APB MDCs, and (b) compares CB and APB PDCs (n = 3).

4.3 Discussion

The secretion of cytokines by DCs influences their maturation, but also the differentiation of Th cells, and differences have been described between MDCs and PDCs present in APB (Almeida et al., 1999). Therefore, the cytokine profile of steady state and stimulated CB MDCs and PDCs was investigated to establish any differences. This involved using the Beadlyte® assay applying Luminex® technology, a recently developed method introduced for investigating multiple cytokine secretion. Other methods include ELISA and intracellular cytokine staining. The ELISA method of detection of cytokine secretion is the most widely used quantitative technique. Unfortunately, this method requires large volumes of supernatant and only a limited number of cytokines can be investigated simultaneously, increasing the cost and time of detection. With intracellular cytokine staining, cells are labelled following stimulation with phytohaemagglutinin (PHA) or phorbol 12-myristate 13-acetate (PMA). This method does not provide a quantitative measurement of cytokine secretion and large cell numbers are required for flow cytometric analysis. The advantages of the Beadlyte® assay using Luminex® technology over other methods include its use of low sample volumes (25 - 50 μl) and its multiplexing capability as up to 100 cytokines can theoretically be investigated simultaneously.

Cytokine secretion is dependent on the choice of stimulus (Edwards., 2002). Therefore, the use of specific stimuli in this study was important to establish the cytokine profile of CB MDCs and PDCs. Initially, IL-3 and IL-4 were added to sustain survival of PDCs and MDCs, respectively, for the detection of steady state production of cytokines. This addition was important for the PDCs, as they do not survive without IL-3 (Grouard et al., 1997). Although MDCs are robust and can survive without the provision of cytokines (Kohrgruber et al., 1999), both cytokines were added to maintain similar conditions. In the steady state, the majority of cytokines were produced at low concentrations. Steady state CB PDCs produced cytokines at levels similar to stimulated PDCs, suggesting that IL-3 was acting as a stimulus in addition to a survival factor. Proinflammatory cytokine production by steady state DCs was supported by another study where immature DCs produced TNFα and IL-6 (Mahnke et al., 2002). Immature DCs also show weak migratory

activities, hence, steady state MDCs secreted low levels of IL-8, a chemokine that directs the migration of cells to sites of inflammation (Jonuleit et al., 1997; Sallusto et al., 2000).

In order to stimulate MDCs and PDCs, LPS and CpG ODN were administered, respectively. Both stimuli were chosen based on the previously reported responses of DCs to them (Hartmann et al., 1999; Kadowaki et al., 2001a). LPS alone has been shown to upregulate CD83 expression and cytokine secretion in APB MDDCs more potently than CD40L or TNFα (Elkord et al., 2005). Also, several studies support the use of CpG as a specific stimulus for PDCs (Hemmi et al., 2000; Bauer et al., 2001b) promoting Th1 differentiation (Adkins, 2000; Cella et al., 2000; Kadowaki et al., 2000), as CD40L is not effective in producing IFNα (Bauer et al., 2001a) instead promoting Th2 responses (Rissoan et al., 1999). The cytokine profiles of CB MDCs and PDCs stimulated with LPS and CpG, respectively, were similar, although reduced concentrations were observed with PDCs, agreeing with other studies (Almeida et al., 1999; Bueno et al., 2001). The pattern of cytokine production was reciprocated with APB MDCs, but not with APB PDCs. The cytokine profile of steady state and CpG-stimulated APB PDCs was restricted to IL-8 and IFNα secretion. As MDCs direct the removal of infection through Th1 responses, it is not surprising that they preferentially secreted proinflammatory cytokines at high concentrations. The significantly reduced concentrations of proinflammatory cytokines secreted by PDCs may suggest a limited role for PDCs in inflammatory reactions.

The difference in cytokine profile observed in this chapter may represent DC subset differences and therefore, the ability of CB MDCs and PDCs to secrete different cytokines following stimulation. IL-10 and IL-12 were solely produced by CB and APB MDCs, and IL-2 and IL-13 by CB PDCs. The production of IL-10 by MDCs may have been due to the choice of stimulus as it has been shown in adults that LPS is a more efficient IL-10 inducer whereas lipotechoic acid induces IL-12 (Hessle et al., 2000). CB MDCs may not be as receptive to LPS for the secretion of IL-10. When DCs are semi-mature, the secretion of cytokines is limited to IL-10 secretion (Kourilsky and Truffa-Bachi, 2001). Therefore, it would be necessary to consider

this for future studies and clinical application. IL-12 was produced at low levels suggesting conversion of DCs from a semi-mature state where IL-10 is produced, to a fully mature DC, where IL-12 production is favoured (Lutz and Schuler, 2002). Another explanation may be the time of administration, as a study has shown a differential cytokine profile when a stimulus was administered immediately compared to addition following 24 hr, suggesting ex vivo manipulation could alter DC signalling (Jiang et al., 2002). Consequently, future studies should be carried out over a 48-hr culture period, varying the time of addition of stimuli as well as the choice of stimuli.

The production of IL-2 by CB PDCs was an unanticipated finding, although, the phenomenon of IL-2 production after bacterial encounter and its involvement in DC-NK interactions has already been described in a murine model (Granucci et al., 2001). IL-2 production by CB DCs may regulate the NK response. IL-2 is also required for the development and survival of T_{regs} (Papiernik et al., 1998; Malek et al., 2002). Therefore, production of IL-2 by PDCs may direct the differentiation of Th cell to T_{regs} resulting in tolerance induction.

Another cytokine solely produced by CB PDCs was IL-13. IL-13 (Rosenzwajg et al., 1998) and IL-2 (Bykovskaja et al., 1998) can be used to expand DC populations from haematopoietic precursors, which would facilitate the development of CB DC subsets. DC derived IL-13 may also inhibit the production of proinflammatory cytokines and indirectly drive naïve T cells towards tolerance or a Th2 pathway, impairing the development of effective Th1 immune responses (de Vries, 1998). GM-CSF is another factor associated with DC differentiation (Santiago-Schwarz et al., 1992) and was also preferentially secreted by CB DC subsets, particularly MDCs. GM-CSF is a cytokine required for the survival of myeloid derived DCs (Palucka et al., 1998). Therefore, its secretion may promote MDC development as shown by *in vitro* studies using GM-CSF and IL-4 to induce MDDC generation.

The production of IFNα by stimulated CB and APB PDCs was in agreement with previous reports (Siegal et al., 1999; Krug et al., 2001a). However, there was a reduced production of IFNα by CB PDCs in comparison to APB PDCs. CB PDCs

may require additional signals to combat viruses. Therefore, through the production of IL-2 and low levels of IFNα, CB PDCs may utilise NK cells to exert cytotoxic activity against viral infection, whereas APB PDCs may combat viruses independently, through the sole production of IFNα. The production of IFNα by CB MDCs was unexpected suggesting a possible bias towards a lymphoid lineage origin. However, viral infection has been shown to induce APB MDCs to produce high levels of IFNα (Diebold et al., 2003), but to my knowledge, the same has not been shown in CB MDCs.

Both MDCs and PDCs from both CB and APB secreted IL-8 following stimulation, a result which is supported by other studies (Rissoan et al., 1999; Penna et al., 2002; Luft et al., 2004). In contrast, production of IL-8 by APB PDCs was limited. IL-8 is produced early in an immune response, but persists for days and even weeks later whereas other cytokines are made and cleared in hours (DeForge et al., 1992). This persistence may explain the abundant production of IL-8 following DC stimulation. The results also suggest that migratory function may be lost by PDCs in adulthood, and MDCs may then become the main cell type to produce IL-8.

The differences in the cytokine profile between CB MDCs and PDCs may be due to differences in the cytokine signalling pathways involving families of molecules such as Janus Activating Kinase (JAK) and Signal Transducer and Activator of Transcription (STAT). It has been shown that there are important differences in the way newborns and adults use STATs to modify immune responses to pathogens (Marodi et al., 2001). Another contributory factor may be the expression of specific cytokine receptors. However, a study has shown that the cytokine receptors on neonatal cells are not expressed at lower levels relative to adult and the receptors do not have a decreased affinity of these receptors to their natural ligand (Marodi et al., 1994). Studying the receptors and the signalling pathways may aid in understanding the function of CB DC subsets compared to their adult counterparts.

The pattern and levels of cytokines produced were important factors to study as they differed between DC subsets and cell sources. For example, physiological concentrations of IL-6 are less than 10 pg/ml in the plasma of adults, but higher in

newborns and a similar result was described in this chapter (Kilpinen et al., 2001; Karlsson et al., 2002). The level of production of cytokines was more variable between APB samples than CB samples, and this may be due to the broad age range of adult donors (17 to 70 years), whereas CB was collected from newborns with a similar gestational age (40 ± 2 weeks). A published study has also detected this variability in cytokine production between individuals (Karlsson et al., 2002). The inconsistency may be important in terms of the regulation and maintenance of cytokine production in individuals. However, regardless of the differences in the levels of cytokine secretion the trend was similar between APB samples.

The results also demonstrate both stimulus and concentration dependencies. A previous study highlighted the importance of the kinetics of DC activation in inducing a particular immune response (Langenkamp et al., 2000). Another study revealed rapid induction of short-lived cytokine responses in DCs following stimulation, particularly with PDCs (Ida et al., 2006). This may explain the apparent lack of cytokine secretion by APB PDCs. Assays that involve stimulation for 5 hr or more may significantly underestimate the cytokine producing capacity of these cells. Therefore, studying further the kinetics of dose response, extending the stimulation times (0 to 72 hr) may reveal further dynamics. However, a problem lies with the lack of sufficient cell numbers to conduct experiments at several time points. Furthermore, delays in the processing of blood can alter the function of DC subsets. This has been shown to some extent with MDCs, but PDC functions were abrogated by this delay probably due to their fragility and need for cytokine or TLR stimulation to maintain survival (Ito et al., 2001).

The results suggested that CB MDCs could produce cytokines as effectively as APB MDCs agreeing with some studies (Karlsson et al., 2002; Upham et al., 2002; Sun et al., 2003), but contradicting other reports of an impaired production of cytokines by CB DCs following stimulation (Stefanovic et al., 1998; Langrish et al., 2002; De Wit et al., 2004). Therefore, the production of cytokines *in vitro* may be influenced by factors present in the culture medium, since AB serum was a component of culture medium used for studies in this thesis. This is supported by a study reporting enhanced TNFα release by newborn monocytes following addition of adult plasma

to washed cells (Levy et al., 2004). Therefore, future studies, replacing the AB serum in culture medium with CB serum may influence the cytokine profile of CB DCs.

The two CB DC subsets respond differentially to specific stimulation in a time-dependent fashion to produce multiple cytokines. Therefore, depending on the DC subset and stimulus, a differential cytokine profile can be generated (Morelli et al., 2001; Karlsson et al., 2002). The results of this chapter indicate flexibility in the cytokine profile of CB DCs, which may be a consequence of their functional immaturity. Certain pathways of cell signalling may be intact in newborns, while others may be defective and non-functioning, and this could be considered for future investigation.

Chapter V

RESULTS 3: FUNCTIONAL CHARACTERISATION OF DENDRITIC CELL SUBSETS

5.1 Introduction

An immune response is initiated following the uptake and processing of foreign antigen by a DC and the subsequent presentation of antigenic peptide to T cells. Immature DCs are efficient in antigen uptake, and internalise pathogens using various mechanisms such as endocytosis. Previous studies have described differences in the mechanisms involved in efficient antigen uptake between MDCs and PDCs (Sallusto et al., 1995). The endocytic capacity of APB DC subsets has been studied (Nijman et al., 1995; Sallusto et al., 1995; Dzionek et al., 2002) along with DCs derived from $CD34^+$ HSCs and $CD14^+$ monocytes isolated from CB (Liu et al., 2001a). However, the process of antigen capture has not been investigated on individual DC subsets from CB. Therefore, in this chapter the endocytic capacity of CB DC subsets was investigated by a flow cytometric assay whereby cells were incubated with soluble FITC-conjugated dextran.

When DCs present foreign MHC molecules to T cells *in vivo* or *in vitro*, a strong T cell proliferative response is initiated. This can be measured by an MLR *in vitro* and by GvHD *in vivo* (Bach and Voynow, 1966; Bosserman et al., 1989; Potolicchio et al., 1996). CB MDDCs have been shown to induce proliferation in an MLR (Liu et al., 2001a), but this has not been established with freshly isolated MDCs or PDCs from CB. Therefore, it was important to study DC-T cell interactions which involved a standard 5-day MLR assay to assess the allostimulatory capacity of both MDCs and PDCs present in CB.

Evidence has also suggested that differences in cytokine secretion patterns following allogeneic stimulation of DCs with T cells play a major role in regulating the immune response (Kalinski et al., 1999). The clinical manifestation of GvHD largely results from cytokine dysregulation (Antin and Ferrara, 1992). Cytokines such as IL-2 and IFNγ promote Th1 immunity and therefore, GvHD (Seder et al., 1993). Cytokines including IL-4, IL-5, IL-6, IL-10 and IL-13 are inducers of a Th2 immune response, reducing the severity of the GvH effect (Abbas et al., 1996). Therefore, following allogeneic stimulation, MLR culture supernatants were obtained to investigate cytokine secretion using the ELISA technique to study IFNγ and IL-4 secretion, and the Luminex® technology for multiple cytokine detection. The assays

were used to measure and compare the functional properties of the CB MDCs and PDCs.

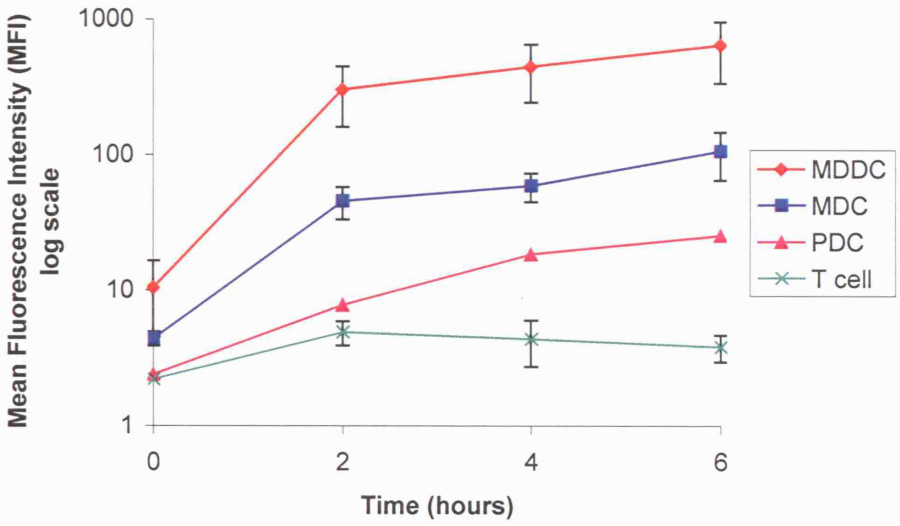
5.2 Results

5.2.1 The endocytic capacity of dendritic cells

The results showed that CB MDCs (Figure 5.1) and APB MDCs (Figure 5.2) had a higher endocytic capacity than PDCs from the same individual, being four-fold more efficient at endocytosis. No peak in endocytosis was reached with either subset within the period examined. The uptake of FITC-dextran was gradual over time for both DC subsets, although initially slightly more rapid for MDCs. The results were represented as mean ± SD of three and four independent experiments with CB (Figure 5.1) and APB cells (Figure 5.2), respectively. T cells were used as the negative control and were unable to endocytose FITC-dextran to significant levels (Figure 5.1a and 5.2a). Conversely, immature MDDCs (Figure 5.1a and 5.2a) were extremely efficient at endocytosis and were used as the positive control. There was a difference in the endocytic capacity between CB and APB MDDCs, with a lower rate of uptake into CB cells, but this difference was not statistically significant ($p > 0.05$). Due to limited cell numbers it was not possible to continue further with longer time points.

The distribution of FITC-dextran following uptake by the various DC subpopulations was also studied. Confocal microscopy highlighted the differential distribution of the FITC-dextran into both MDDCs and MDCs at the 6 hr time point (Figure 5.3). The uptake was best represented with MDDCs (Figure 5.3a), as these cells were highly efficient at endocytosis as previously demonstrated (Figure 5.1a and 5.2a). The image clearly showed a greater uptake of soluble molecules and a wider distribution when compared to MDCs (Figure 5.3). The intensity of the FITC-dextran was also greater in MDDCs compared to MDCs. Visible uptake of FITC-dextran by the PDC population was apparent, but due to the bleaching of the fluorescence an image could not be captured. The representative images were similar for both CB and APB supporting the FACS data.

(a)



(b)

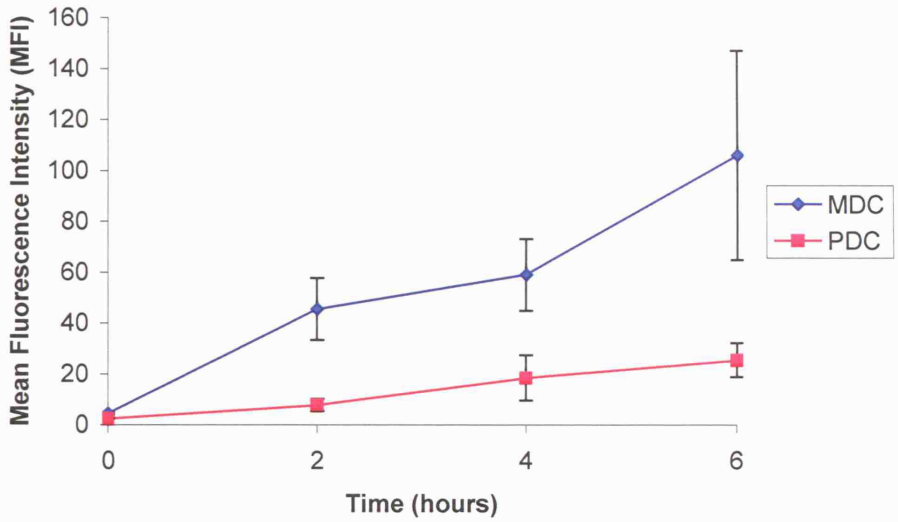
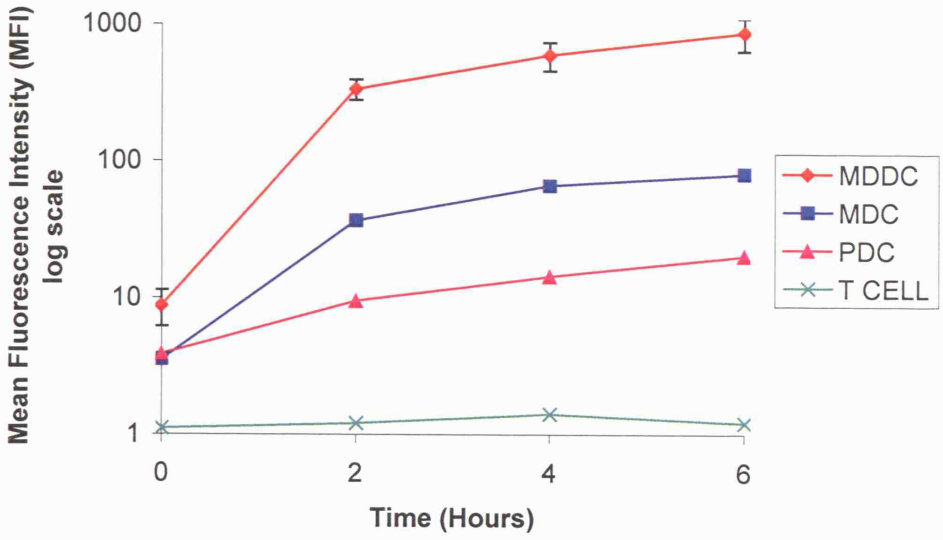


Figure 5.1. The endocytic capacity of CB MDCs and PDCs.

Different cells isolated from CB were treated with FITC-dextran and endocytic capacity assessed at various time points. The uptake was then analysed by flow cytometry, and data expressed as mean fluorescence intensity (MFI). (a) the endocytic capacity of MDCs and PDCs were studied with MDDCs included as the positive control and T cells as a negative control. The graph is represented in a logarithmic format. (b) represents the same data, re-plotted to illustrate the difference between the MDC and PDC populations. The results for each set of experiments are the mean ± SD of three independent experiments (n = 3).

(a)



(b)

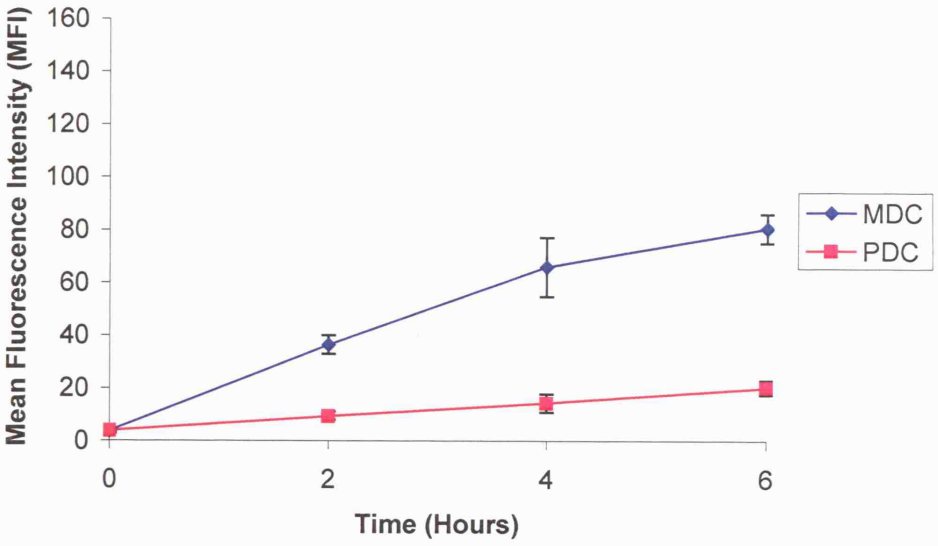
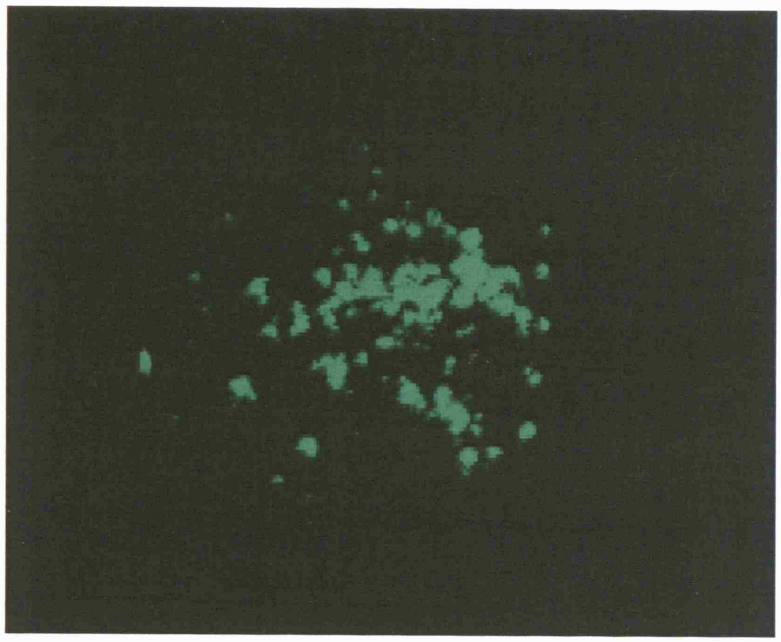


Figure 5.2. The endocytic capacity of APB MDCs and PDCs.

Different cells isolated from APB were treated with FITC-dextran and endocytic capacity assessed at various time points by flow cytometry, and data expressed as mean fluorescence intensity (MFI). (a) the endocytic capacity of MDCs and PDCs were studied with MDDCs as the positive control and T cells as a negative control. The graph is represented in a logarithmic format. (b) represents the difference between the MDC and PDC populations. The results for each set of experiments are the mean ± SD of four independent experiments (n = 4).

(a)



(b)

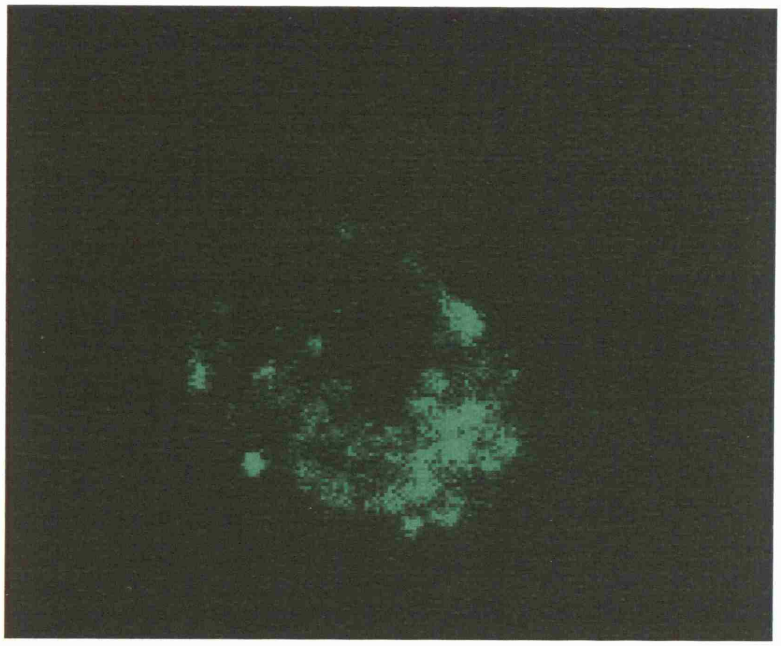


Figure 5.3. Confocal images of the endocytic capacity of myeloid derived dendritic cells.

The distribution of FITC-dextran in (a) MDDCs and (b) MDCs, following a 6 hr incubation period. The three-dimensional images were obtained using confocal microscopy.

5.2.2 The allostimulatory capacity of dendritic cells

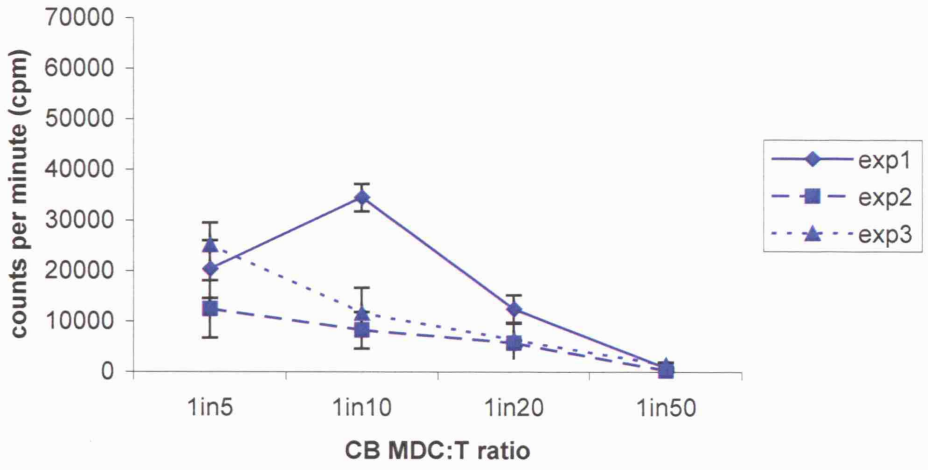
The allostimulatory capacity of CB DC subsets was assessed using the standard 5-day MLR assay following isolation of cells using the system of magnetic columns. For these assays, HLA typing was performed on all samples investigated to confirm mismatching at the HLA level to mimic and further understand the *in vivo* T cell proliferative response of GvHD. All results show the mean ± SD of at least three independent experiments with triplicates for each set of experiments.

5.2.2.1 MLR using fresh cells

The isolated DC subsets were co-cultured for 5 days with HLA disparate allogeneic $CD3^+$ T cells isolated from APB before ^3H Thymidine incorporation. The stimulator (DC subset) to responder (T cell) populations were cultured at ratios of 1:5, 1:10, 1:20 and 1:50.

When comparing DC subsets, MDCs had a significantly greater allostimulatory capacity than PDCs ($p < 0.05$) (Figure 5.5. and 5.5). In the CB setting, there were comparable results for MDCs and PDCs with both DC subsets being efficient stimulators of an allogeneic response (Figure 5.6). A lower allostimulatory capacity was observed with MDCs from CB than the corresponding cells from APB. However, this was reversed in the CB setting of PDCs where CB PDCs were better stimulators than their adult counterparts. Hence, the difference in allostimulatory capacity between APB MDCs and PDCs was more pronounced. An increase in DC-T cell ratio resulted in a decrease in the allostimulatory capacity of both MDCs and PDCs.

(a)



(b)

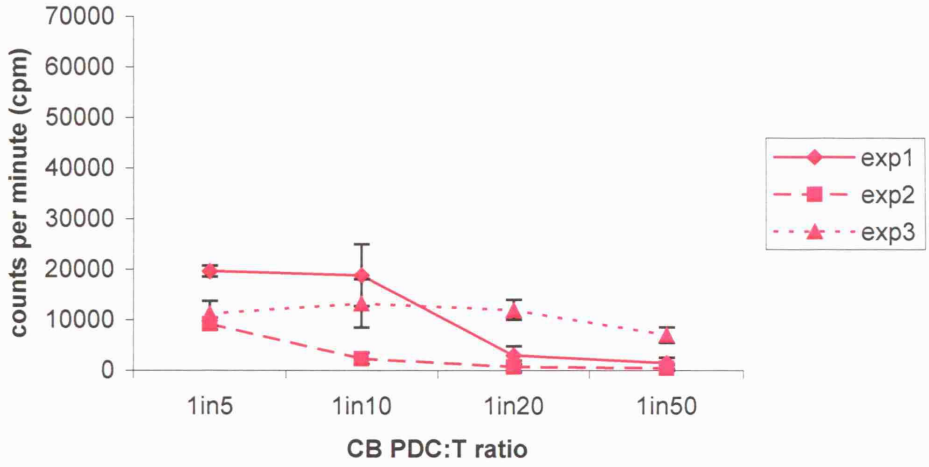
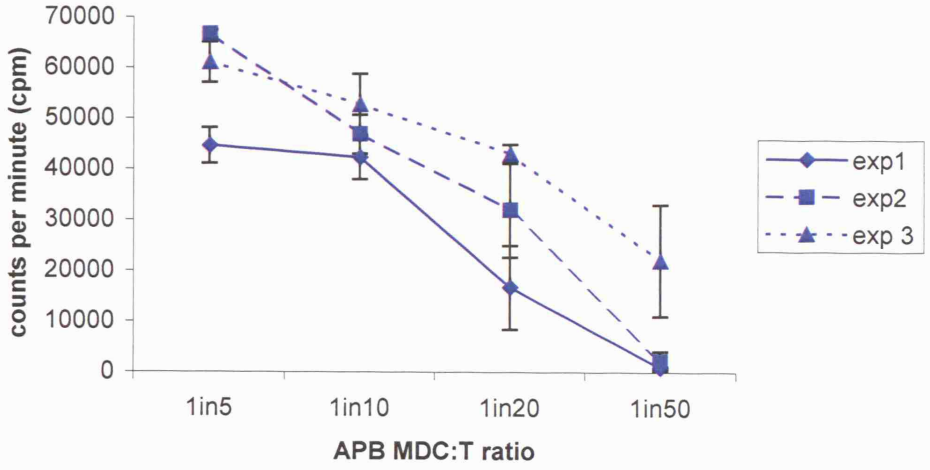


Figure 5.4. The allostimulatory capacity of fresh CB MDCs and PDCs.

The allostimulatory function of (a) MDCs and (b) PDCs freshly isolated from CB in a primary allogeneic MLR. DC subsets were co-cultured with APB allogeneic T cells and the proliferative response was measured after 5 days using ^{3}H Thymidine incorporation. Values of each independent experiment are the mean ± SD obtained from triplicate cultures (n = 3).

(a)



(b)

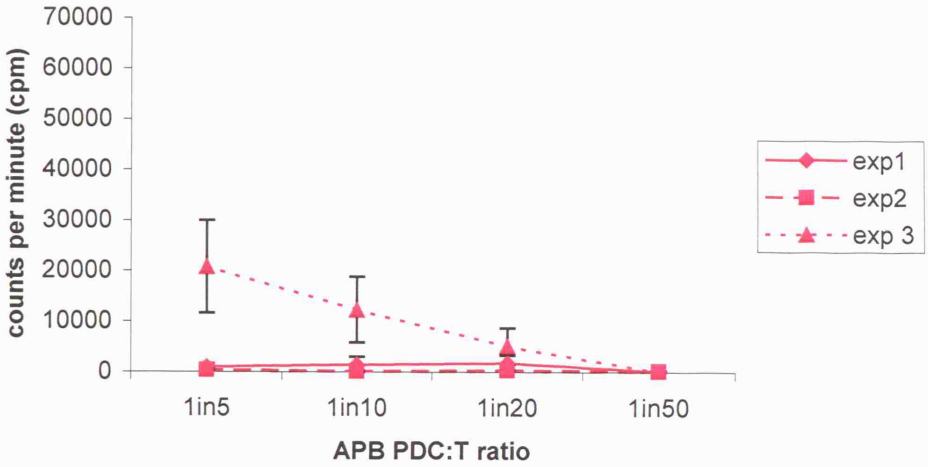


Figure 5.5. The allostimulatory capacity of fresh APB MDCs and PDCs.

The allostimulatory function of (a) MDCs and (b) PDCs freshly isolated from APB in a primary allogeneic MLR. DC subsets were co-cultured with APB allogeneic T cells and the proliferative response was measured after 5 days using ^{3}H Thymidine incorporation. Values of each independent experiment are the mean ± SD obtained from triplicate cultures (n = 3).

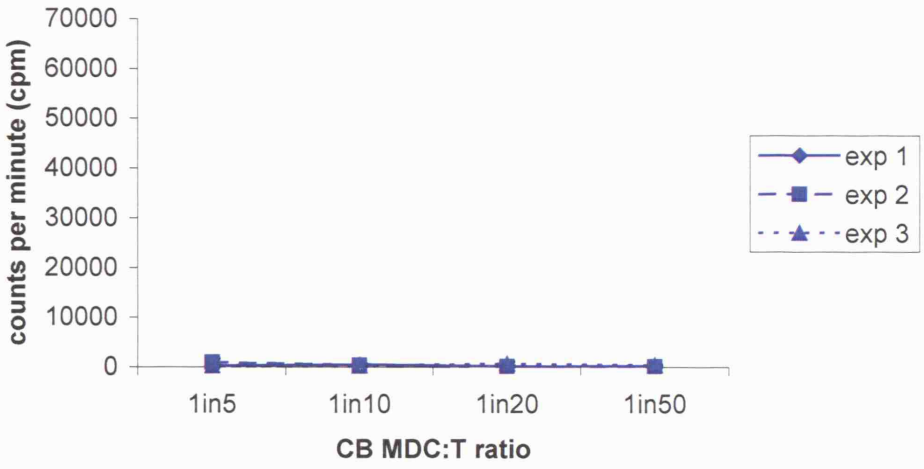
5.2.2.2 MLR using frozen-thawed cells

MLR assays were carried out on DC subsets following the freeze-thaw process to mimic the state of cryopreserved CB units. DC subsets were isolated from frozen-thawed MNCs following viability assessment by trypan blue staining. Viability of the MNCs was always in the region of 90-95%.

The allostimulatory capacities of frozen CB MDCs and PDCs were similar, although, CB PDCs were slightly better stimulators than CB MDCs (Figure 5.6 and Appendix 4). The allostimulatory capacity of MDCs and PDCs isolated from frozen CB MNCs was significantly reduced compared to fresh cells ($p < 0.05$) (Figure 5.4 and 5.6).

When frozen MNCs were used to isolate APB MDCs and PDCs, the results paralleled those of fresh APB cells with the highest allostimulatory capacity detected with MDCs (Figure 5.5 and 5.7).

(a)



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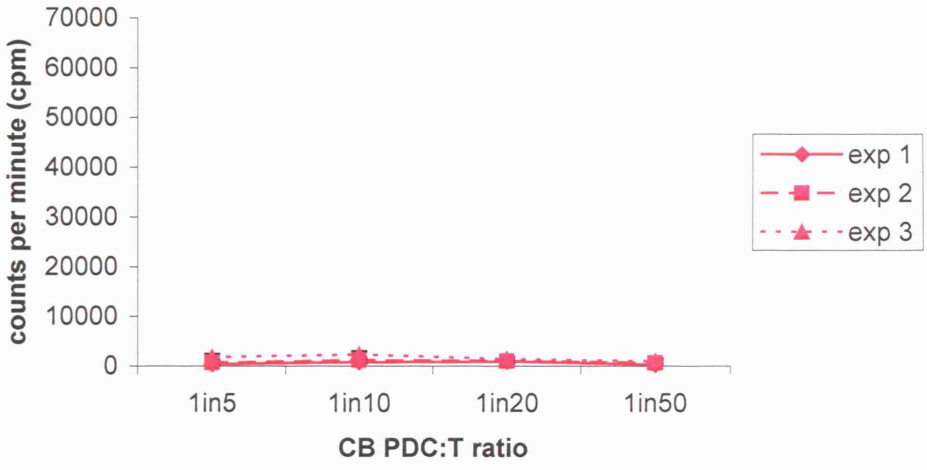
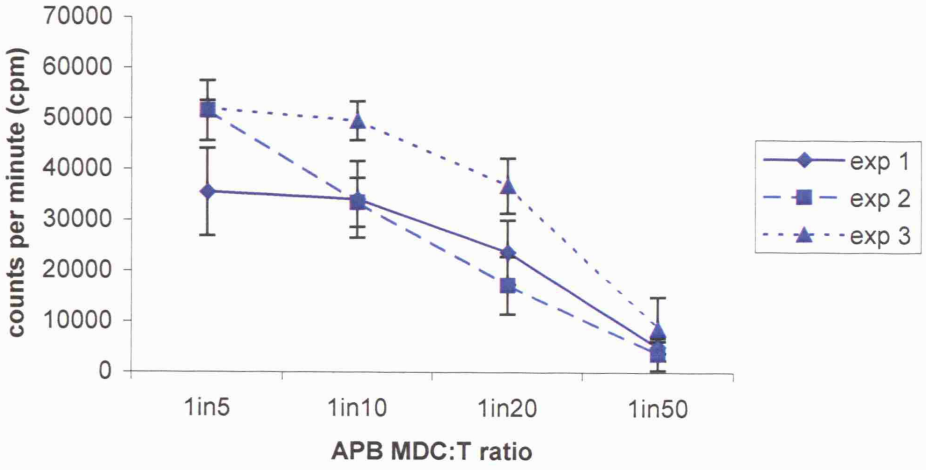


Figure 5.6. The allostimulatory capacity of frozen CB MDCs and PDCs.

The allostimulatory function of (a) MDCs and (b) PDCs isolated from frozen CB MNCs in a primary allogeneic MLR. DC subsets were co-cultured with APB allogeneic T cells and the proliferative response was measured after 5 days using ^{3}H Thymidine incorporation. Values of each independent experiment are the mean ± SD obtained from triplicate cultures (n = 3).

(a)



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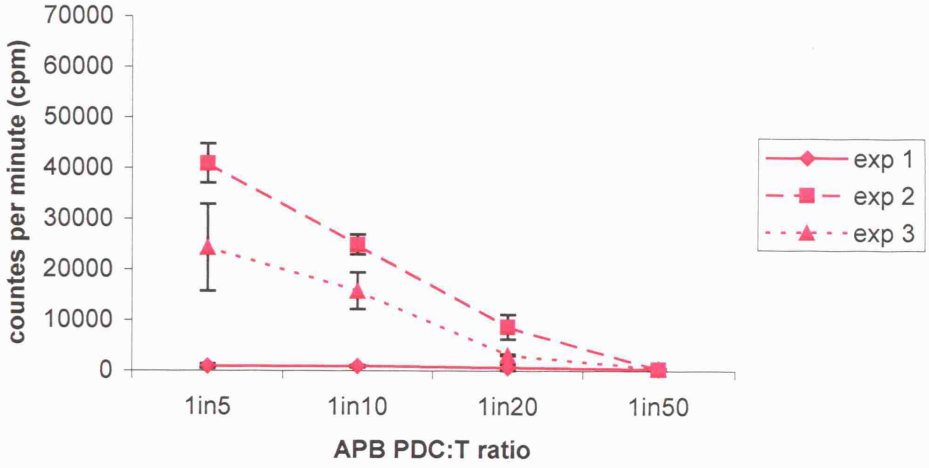


Figure 5.7. The allostimulatory capacity of frozen APB MDCs and PDCs.

The allostimulatory function of DC subsets isolated from frozen APB in a primary allogeneic MLR. DC subsets were co-cultured with APB allogeneic T cells and the proliferative response was measured after 5 days using ^{3}H Thymidine incorporation. Values of each independent experiment are the mean ± SD obtained from triplicate cultures (n = 3).

5.2.3 *Cytokine secretion following an MLR*

Following allogeneic stimulation of CB DC subsets with T cells, cytokine secretion was studied using the methods of ELISA and Luminex® technology.

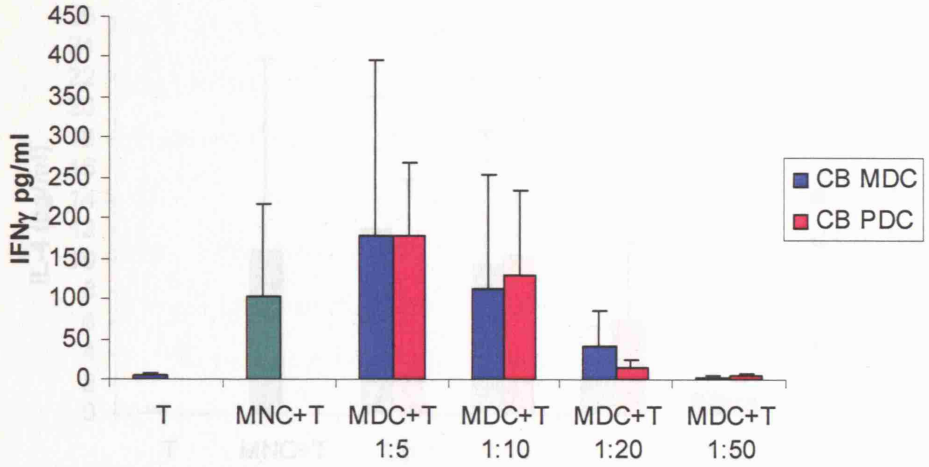
5.2.4 *Secretion of IFNγ*

The production of IFNγ following allostimulation was equivalent between CB MDCs and PDCs (Figure 5.8a). The ratio of 1:20 signalled a dramatic reduction in the levels of IFNγ production and at a 1:50 ratio levels were similar to the negative control of T cells alone. When APB MDCs were the designated stimulator population, the production of IFNγ significantly increased compared to APB PDCs (Figure 5.8b). However, a decrease in the number of DC subsets used in the MLR was accompanied by a reduction in the amount of IFNγ produced.

5.2.5 *Secretion of IL-4*

Levels of secreted IL-4 were similar between MDCs and PDCs, with low levels detected (Figure 5.9). When comparing IL-4 production in terms of CB and APB, differences were only noted at the higher ratios (1:20 and 1:50). At these ratios, production of IL-4 was lower in the cord setting using MDCs compared to APB (Figure 5.9). The use of CB PDCs as stimulators resulted in the production of higher levels of IL-4 compared to APB PDCs (Figure 5.9).

(a)



(b)

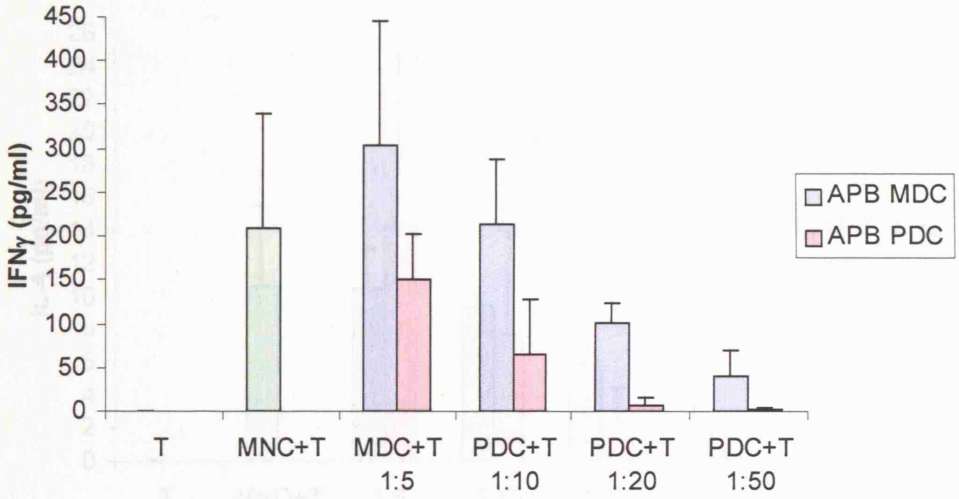
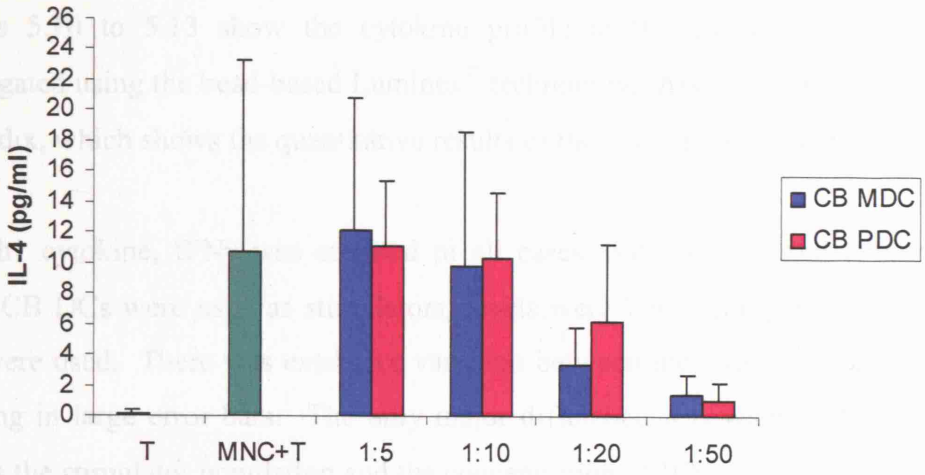


Figure 5.8. The production of IFNγ following allogeneic stimulation with MDCs and PDCs.

Following isolation the DC subsets were co-cultured with allogeneic T cells for 5 days after which time culture supernatants were removed to investigate the production of IFNγ by ELISA. The graphs indicate cytokine secretion when either (a) MDCs or (b) PDCs were used as stimulators. The results show the mean ± SD of three independent experiments (n = 3) denoted by ■ for CB cells and ≡ for APB cells. ■ represents the control, ■ represents MDCs, and ■ is for PDCs.

Note: The source of MNCs was CB when CB DCs were the stimulators and APB for APB DCs.

(a)



(b)

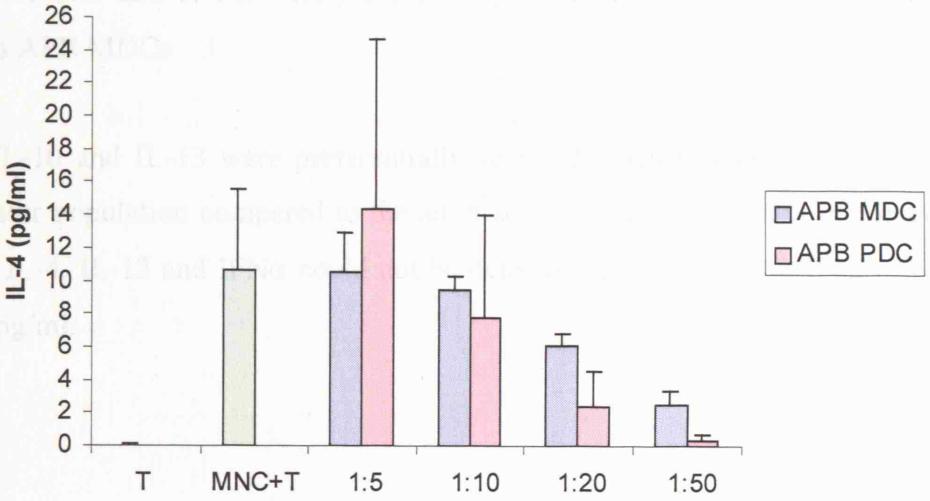


Figure 5.9. The production of IL-4 following allogeneic stimulation with MDCs and PDCs.

Following isolation the DC subsets were co-cultured with allogeneic T cells for 5 days after which time culture supernatants were removed to investigate the production of IL-4 by ELISA. The graphs indicate cytokine secretion when either (a) MDCs or (b) PDCs were used as stimulators. The results show the mean ± SD of three independent experiments (n = 3) denoted by ■ for CB cells and ≡ for APB cells. ■ represents the control (CB or APB MNCs + APB T cells), ■ represents MDCs, and ■ is for PDCs.

5.2.6 Multiple cytokine detection

Figures 5.10 to 5.13 show the cytokine profile at the DC-T cell ratio of 1:10 investigated using the bead-based Luminex® technology. Also, refer to table 5.1 of the Appendix, which shows the quantitative results of the cytokine secretion.

The Th1 cytokine, IFNγ was secreted in all cases, but levels varied (Figure 5.10). When CB DCs were used as stimulators, levels were lower compared to when APB cells were used. There was extensive variation between individuals in the CB setting resulting in large error bars. The only major difference was when APB PDCs were used as the stimulator population and the concentration of IFNγ was reduced compared to APB MDCs and CB ($p < 0.05$).

IL-8, GM-CSF and TNFα were preferentially secreted following allostimulation with CB and APB MDCs

IL-6, IL-10 and IL-13 were preferentially secreted when CB DCs were used as the stimulator population compared to the adult setting. The production of the cytokines IL-1β, IL-4, IL-12 and IFNα could not be detected above the lower limit of detection of 6.9 pg/ml.

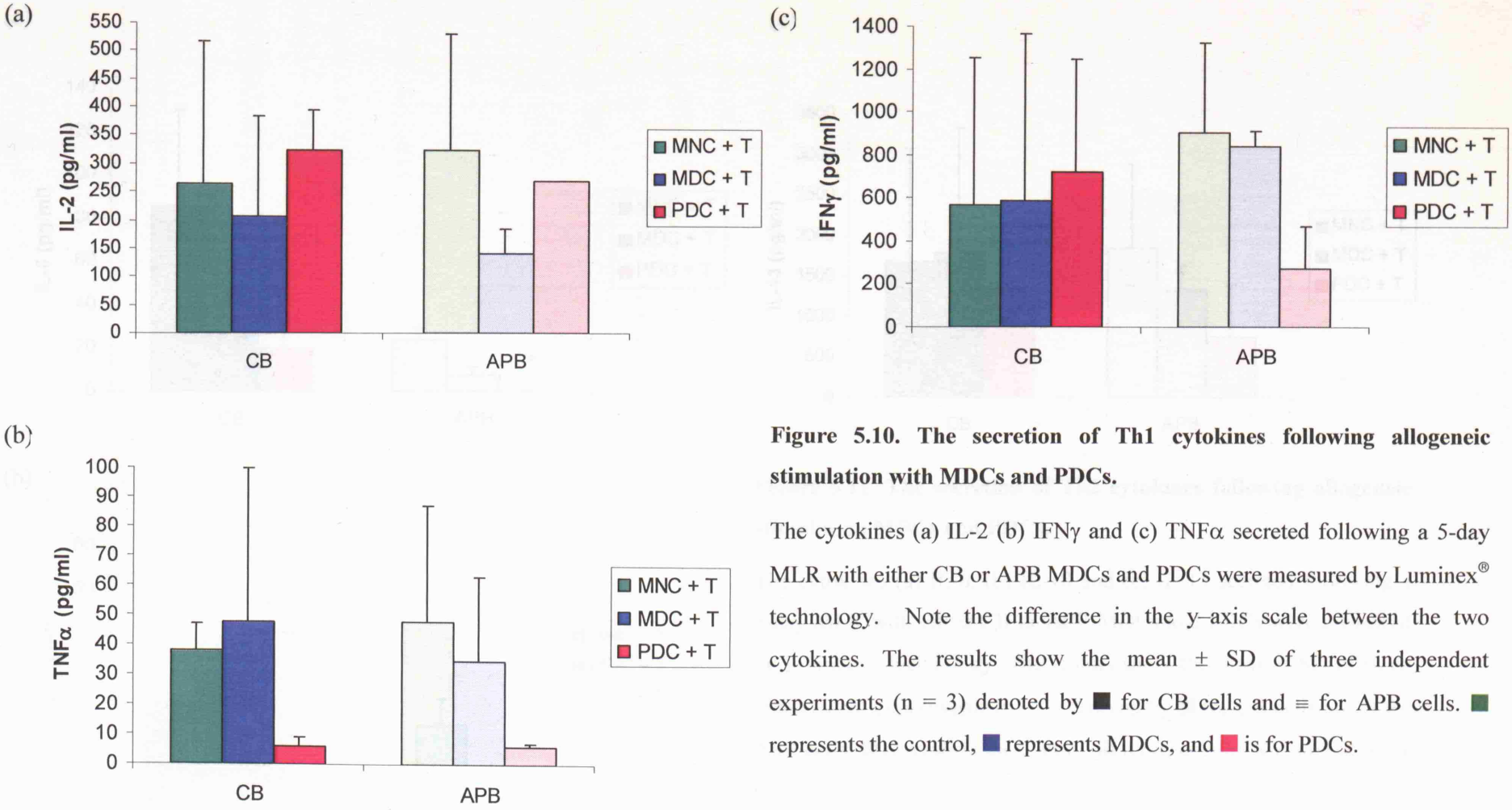
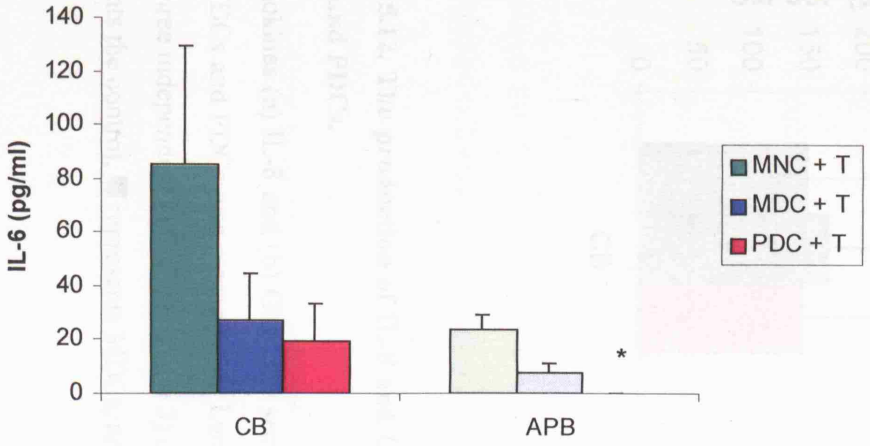


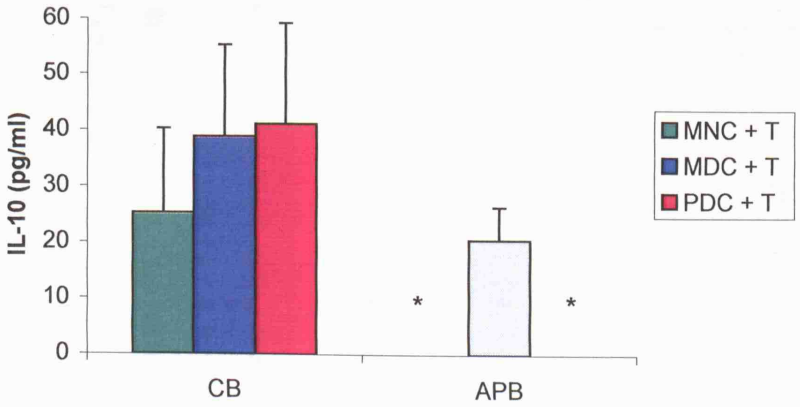
Figure 5.10. The secretion of Th1 cytokines following allogeneic stimulation with MDCs and PDCs.

The cytokines (a) IL-2 (b) IFNγ and (c) TNFα secreted following a 5-day MLR with either CB or APB MDCs and PDCs were measured by Luminex® technology. Note the difference in the y–axis scale between the two cytokines. The results show the mean ± SD of three independent experiments (n = 3) denoted by ■ for CB cells and ≡ for APB cells. ■ represents the control, ■ represents MDCs, and ■ is for PDCs.

(a)



(b)



(c)

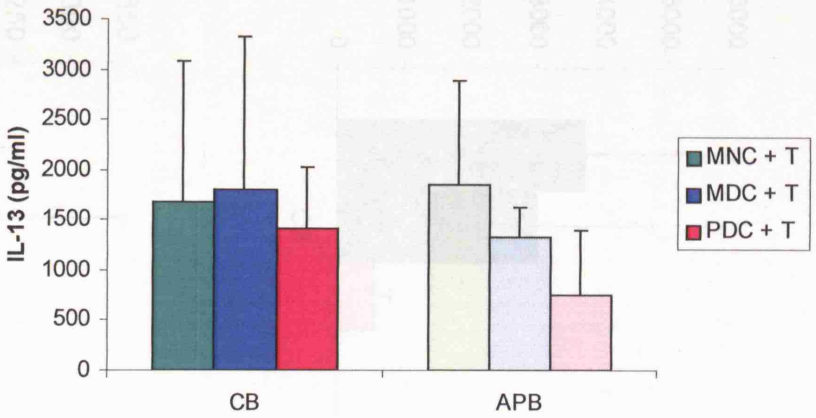
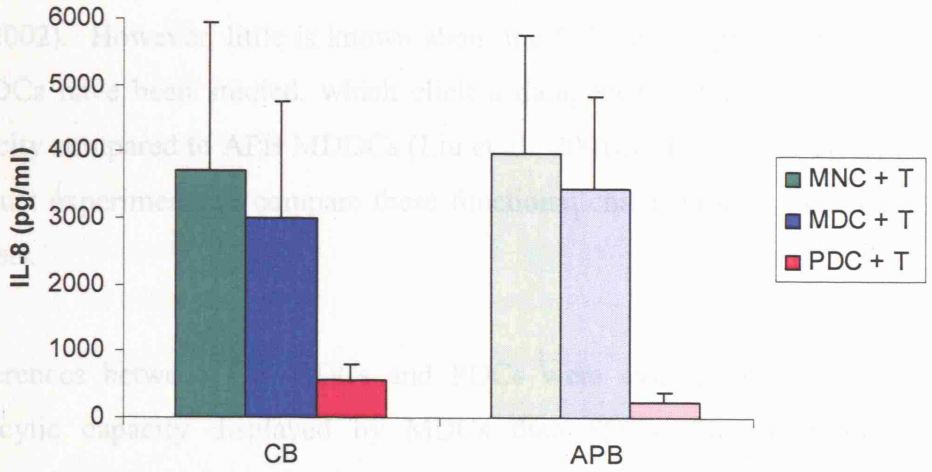


Figure 5.11. The secretion of Th2 cytokines following allogeneic stimulation MDCs and PDCs.

The cytokines (a) IL-6, (b) IL-10 and (c) IL-13 secreted following a 5-day MLR with either CB or APB MDCs and PDCs were measured by Luminex® technology. The results show the mean ± SD of three independent experiments (n = 3) denoted by ■ for CB cells and ≡ for APB cells. ■ represents the control, ■ represents MDCs, and ■ is for PDCs.

(* = below the level of detection)

(a)



(b)

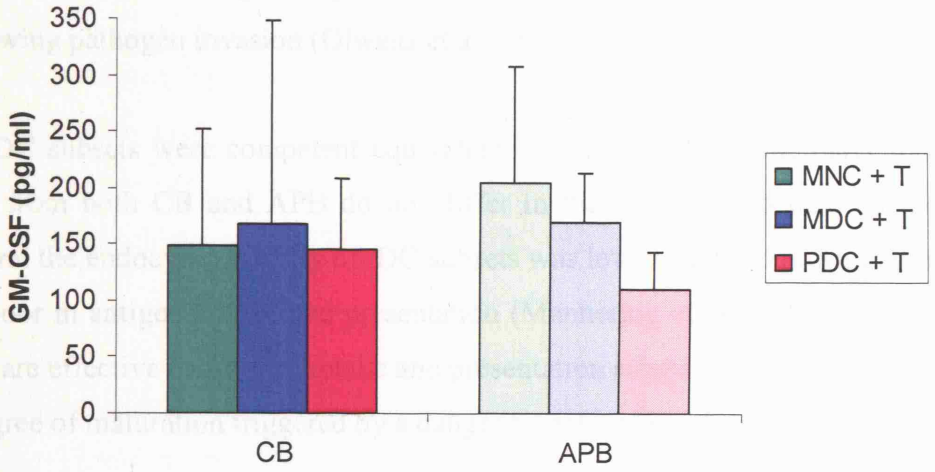


Figure 5.12. The production of IL-8 and GM-CSF following allogeneic stimulation with MDCs and PDCs.

The cytokines (a) IL-8 and (b) GM-CSF secreted following a 5-day MLR with either CB or APB MDCs and PDCs was measured by Luminex® technology. The results show the mean ± SD of three independent experiments (n = 3) denoted by ■ for CB cells and ≡ for APB cells. ■ represents the control, ■ represents MDCs, and ■ is for PDCs.

5.3 Discussion

The differences in both the endocytic and allostimulatory capacity between MDCs and PDCs isolated from APB have been demonstrated (Dzionek et al., 2000; MacDonald et al., 2002). However, little is known about the DC subsets present in CB as only CB MDDCs have been studied, which elicit a dampened endocytic and allostimulatory capacity compared to APB MDDCs (Liu et al., 2001a). It was, therefore, important to conduct experiments to compare these functional characteristics of CB and APB DC subsets.

Differences between CB MDCs and PDCs were evident with a four-fold higher endocytic capacity displayed by MDCs than PDCs. This may be a factor in determining the number of antigens presented to T cells, with MDCs presenting more antigens compared to PDCs. PDCs may migrate to lymphoid tissues independently of inflammation and foreign antigens, and hence become unavailable for antigen uptake following pathogen invasion (Olweus et al., 1997).

CB DC subsets were competent equivalents to their adult counterparts showing that DCs from both CB and APB do not differ in their abilities to take up antigen. In general the endocytic capacity of DC subsets was low, and it is known that blood DCs are poor in antigen uptake and presentation (Mannering et al., 1998). Cultured blood DCs are effective in antigen uptake and presentation suggesting that blood DCs require a degree of maturation triggered by a danger signal, to be active for antigen uptake.

The findings that the allostimulatory capacity of fresh CB MDCs was greater than CB PDCs but lower than APB MDCs clearly demonstrate the functional impairment of CB MDCs. A reduced allostimulatory capacity of CB MDCs is most likely a consequence of the reduced levels of expression of HLA-DR molecules on CB MDCs (see chapter III). The dampening of a Th1 immune response using CB MDCs and the prevalence of PDCs in CB may override Th1 immunity to induce Th2 immunity or tolerance (Kuwana et al., 2001). The results reinforce the fact that neonatal immunity is biased against Th1 immunity (Langrish et al., 2002).

Due to time constraints, CB T cells were not used as responders and this may have influenced the outcome of the results. Therefore, the introduction of CB T cells may show a further reduction in the allostimulatory capacity of CB MDCs, suggesting CB DC subsets are functionally immature cells. In CBT, the naivety of CB T cells in combination with immature CB DCs would be the ideal setting for transplantation tolerance or Th2 immunity.

The allostimulatory capacity of frozen CB and APB DCs was also investigated to determine the effect of freezing and thawing since all CB units are cryopreserved prior to transplantation. Following the freeze-thaw process, the allostimulatory capacity of CB MDCs and PDCs was significantly reduced compared to fresh DC subsets. It is possible that frozen cells may have lost their functional capabilities, becoming inactivated, or undergoing apoptosis during culture as cells were viable at the initiation of culture. The possibility of a technical failure following thawing may be feasible and this may be studied in future to ascertain the viability of DCs at various stages (following thawing and then isolation) prior to culture. Other data has shown different outcomes with regards to the function of fresh and frozen DCs, some revealing functionally deficient DCs and others with a function equivalence following the freeze-thaw process (Harris et al., 1994; Sato et al., 1998; Lewalle et al., 2000; Hori et al., 2004).

The allostimulatory capacity of frozen-thawed APB MDCs was similar to that of fresh MDCs. However, the allostimulatory capacity of frozen APB PDCs was elevated compared to fresh cells agreeing with another study, suggesting the freezing process resulted in partial maturation (John et al., 2003). However, other studies suggest that frozen-thawed DCs are phenotypically and functionally comparable to fresh DCs (Lewalle et al., 2000; Hori et al., 2004), which could explain the results of the allostimulatory capacity of MDCs. Therefore, studying the markers of maturation would be valuable in determining the status of APB DC subsets following the freeze-thaw process.

Following allogeneic stimulation culture supernatants were removed for the detection of cytokines. The two cytokines initially selected for investigation by ELISA were IFNγ and IL-4. The production of IFNγ was similar between CB MDCs and PDCs as

stimulators, but lower than the adult setting, particularly with MDCs. CB DC stimulation inducing IFNγ secretion suggests that CB DCs have the capability to function as effectively as adult cells and their environment may influence their actions. IL-4 production was similar between DC subsets and between CB and APB. The substantial production of IFNγ and very little IL-4 secretion by T cells following allogeneic stimulation may be due to the regulation of IL-4 expression in T cells by IFNγ in a dosage specific manner (Elser et al., 2002). The results suggest that the Th1 immune response is not dampened by IL-4 secretion (O'Garra, 1998). Therefore, it will be interesting to study the allostimulatory capacity using CB T cells as the responder population.

To gain a broader view of cytokine secretion patterns, the bead-based multiplexing method of cytokine detection was used to study the secretion of proinflammatory, Th1 and Th2 inducing cytokines following allogeneic stimulation. IL-6 and IL-10 were produced at greater levels following stimulation with CB DCs compared to APB DCs, which is consistent with another study for IL-10 (Rainsford and Reen, 2002). The preferential production of IL-6 and IL-10 may be important in the derivation of Th2 and T_{regs} cells, respectively (Levings et al., 2001; McKee and Pearce, 2004). Both IL-6 and IL-10 inhibit the production of the pro-inflammatory cytokines, IL-1 and TNFα, (Aderka et al., 1989; Schindler et al., 1990; Moore et al., 1993) which are thought to contribute to the GvH effect as they are secreted simultaneously during BMT pre-conditioning (Xun et al., 1994). The relationship between IL-10 production and reduced GvHD is evidenced in several reports proposing that abundant IL-10 production may be a regulator of increased tolerance (Krenger et al., 1994; Groux et al., 1996; de, V et al., 1998; Rainsford and Reen, 2002). IL-6 and IL-10 also share common signalling mechanisms of STAT3 activation (Lai et al., 1996; Wehinger et al., 1996). The above factors may indicate why IL-6 and IL-10 were produced simultaneously.

Other factors in the microenvironment may also dictate the production of cytokines directing Th1 or Th2 responses (Vieira et al., 2000). A study showed that Th2 polarisation results if adult or neonatal DCs are transferred into neonates. However, when transferred into adult recipients, neonatal DCs elicit CTL and Th1 responses

(Sun et al., 2005). Therefore, the environment in which DCs are present may contribute to the impaired Th1 response, and Th2 skewing of neonatal immunity. In this thesis, DCs were cultured in medium containing AB serum which may have influenced cytokine production. Investigating cytokine production following culture in CB serum may alter the cytokine profile.

The results showed that CB MDCs had a higher endocytic capacity compared to PDCs, and CB DC subsets are equivalent to APB DCs with regards to their endocytic capacity. The allostimulatory capacity of CB MDCs was also higher than PDCs but lower than APB MDCs, and was further reduced by their susceptibility to freezing and thawing reinforcing their immature status. Freezing may further render them susceptible to environmental factors. CB DCs may be more functionally flexible than APB DCs since they are in the process of development. CB DCs may also preferentially direct the secretion of Th2 inducing cytokines. Conversely, APB DCs may have a more limited functional capacity inducing the secretion of Th1 cytokines, due to their developed status.

Chapter VI

GENERAL DISCUSSION

GvHD is a serious complication of haematopoeitic stem cell transplantation mediated by immunocompetent donor T cells recognising the histoincompatible antigens in the recipient as foreign through the direct and/or indirect pathway of allorecognition (Murphy and Blazar, 1999). During direct allorecognition, alloantigens on recipient DCs are recognised by donor T cells (Shlomchik et al., 1999), whereas the indirect pathway involves donor T cells recognising recipient-derived peptides on donor DCs (Baker et al., 2001). The direct pathway of allorecognition has been established as the primary mechanism for *a*GvHD development (Shlomchik et al., 1999; Sato et al., 2003). Although donor blood DCs were found in patients with *c*GvHD (Clark et al., 2003), the role of donor DCs in *a*GvHD has only recently been described (Matte et al., 2004). The relative contribution of recipient and donor DCs to the pathway of allorecognition may be important in the pathogenesis of GvHD (Lechler et al., 2001). Studies using CB as a HSC source have shown a reduction in the incidence and severity of GvHD (Gluckman et al., 1997; Rocha et al., 2000). Although the precise immunological mechanisms are not yet known, and since T cells are directly implicated in GvHD, one of the possible explanations for the reduced incidence of GvHD following CBT is the presence of naïve T cells present in CB compared to APB or BM. In addition, CB T cells produce less IL-2, IFNγ and IL-4 than APB (Bofill et al., 1994; Chalmers et al., 1998). However, it could also be argued that since DCs are involved in the presentation of antigen via the direct and indirect pathway, it is possible that their immunological and developmental stages may also influence the reduced GvHD observed following CBT. Therefore, this thesis has attempted to address this question by studying the DC subsets present in CB and comparing their phenotypic and functional characteristics with those present in APB.

6.1 DC subset prevalence in cord blood

The absolute cell counting results confirmed the differential subset distribution of MDC versus PDC in CB where PDCs predominate over MDCs. In contrast, the predominant DC subpopulation in APB is the MDC (see Chapter III; Sorg et al., 1999; Borras et al., 2001). Investigators have reported that differences in the prevalence of DC subsets may account for diminished antigen presenting

of CB cells (Sorg et al., 1999; Borras et al., 2001; Drohan et al., 2004). In addition, MDCs have been shown to induce a Th1 immune response whereas, PDCs direct Th2 immunity (Rissoan et al., 1999; Liu et al., 2000). In the HSCT context, a Th1 response secreting inflammatory cytokines, IL-2 and IFNγ, would be detrimental to the recipient following transplantation, since it could contribute to the development of GvHD. The high PDC count in CB may instead direct Th2 polarisation through secretion of IL-4, IL-5 and IL-10 which may dampen the Th1 response, hence reduce the severity of GvHD. A study involving the transplantation of a heart into a murine model showed that donor PDCs infused into the recipient inhibited proliferative responses and no rejection was observed (O'Connell et al., 2002). A correlation with the reduced occurrence of GvHD and the presence of PDCs, originating from either the graft (Liu and Blom, 2000; Rossi et al., 2002) or host HSCs (Arpinati et al., 2000; Teshima et al., 2002b) has also been described. Further clinical studies have revealed that development of GvHD may be a consequence of reduced numbers of circulating PDCs (Mohty et al., 2005). However, it is important to note that increased numbers of PDCs have been associated with *c*GvHD (Rossi et al., 2002; Clark et al., 2003).

The results of this thesis showing significantly increased PDC numbers in CB compared to APB (see Chapter III) is supported by studies where PDC counts are higher in children in whom the incidence of GvHD is reduced when compared with adults (Klingebiel and Schlegel, 1998; Teig et al., 2002; Teig et al., 2002; Vakkila et al., 2004). Therefore, due to the various factors that promote or inhibit GvHD, it is important to maintain a balance between the numbers of DC subsets in a graft in order to induce tolerance or immunity, preventing GvHD but also encouraging engraftment, GvL and a lower rate of relapse.

Clinical approaches to limit GvHD occurrence have included Campath-1G treatment to deplete host DC numbers, or the use of Flt3L prior to transplantation in murine models, to expand host $CD8\alpha^+$ DCs (Klangsinsirikul et al., 2002; Teshima et al., 2002b). As several pieces of evidence suggest that donor DCs present in the graft also influence the outcome of HSCT, strategies to prevent GvHD included the administration of Granulocyte-Colony Stimulating Factor (G-CSF) to donors to

expand the PDC population (Arpinati et al., 2000). G-CSF is known to expand PDCs derived from PBSCs without altering MDC numbers, leading to the induction of Th2 differentiation associated with a tolerogenic immune response *in vitro,* and resulting in prolonged graft survival *in vivo* (Arpinati et al., 2000). In the CBT setting, administration of factors to increase numbers may not be necessary since PDCs are preferentially present in CB. The predominance of PDCs in CB may suggest that the proposed actions for initiating GvHD following CBT may be altered and instead of DCs inducing immunity, they may direct the T cells towards Th2 immunity or tolerance.

1.2 Phenotypic immaturity of CB DC subsets

Immunophenotyping results revealed a similar expression of a range of markers on CB and APB DCs, but CB DCs showed a reduced intensity of expression compared to APB DCs agreeing with published data (Hunt et al., 1994; Petty and Hunt, 1998; Sorg et al., 1999). The results also showed that markers of immature/precursor cells such as CD34, CD45RA and CD133, were preferentially expressed by CB DC subsets. PDCs preferentially expressed CD34, and MDCs were positive for CD133. The latter result may be supported by a study which has shown that CB $CD133^{+}CD34^{-}$ cells, positive for CD13 and CD33 antigens, differentiate into DC precursors with myeloid characteristics (Goussetis et al., 2006). It could be speculated that $CD133^{-}CD34^{+}$ cells could develop into PDCs. However, $CD133^{-}$ $CD34^{+}$ cells have been shown to be committed to B cell and erythroid lineages, whereas, $CD133^{+}CD34^{+}$ cells represent primitive and myeloid progenitors (Yin et al., 1997; Bhatia, 2001). CB DC subsets may represent a differential stage of development or alternatively, a completely separate lineage compared to APB DCs. Future experiments including DC subset differentiation from $CD133^{+}$ and $CD34^{+}$ stem cells should be carried out to identify lineage commitment.

Another molecule known to be expressed by early stem cells is the nonclassical HLA class I molecule, HLA-G. The results from this thesis showed that CB DC subsets expressed HLA-G mRNA transcripts, which were detected at a higher intensity in CB MDCs compared to PDCs or APB MDCs (see Chapter III, Figure

3.7). The preferential expression of the membrane isoform, HLA-G1 and the soluble isoform, HLA-G5, in CB MDCs, may have contributed to the reduction in the allostimulatory capacity of CB MDCs compared to APB MDCs (see Chapter VI), as membrane bound HLA-G1 has been shown to suppress $CD4^+$ T cell proliferation and soluble HLA-G1 (i.e. HLA-G5) can induce the apoptosis of $CD8^+$ T cells (Bainbridge et al., 2000a; Le Bouteiller and Solier, 2001). However, further experiments need to be performed in order to assess this possibility. The immunosuppressive properties of HLA-G may be important in dampening the Th1 response, possibly leading to tolerance induction. HLA-G expression is also dependent on the activating stimuli in the microenvironment where its expression is upregulated when monocytes are exposed to IL-10 (Moreau et al., 1999). Therefore, secretion of IL-10 into the DC environment may induce HLA-G expression, which in turn suppresses an immune response. HLA-G may have other consequences regarding the function of NK cells, T cells, and DCs themselves, on the innate and acquired immune response developed in malignant, inflammatory, and post-transplantation conditions.

The results obtained regarding the expression of molecules involved in the HLA class II antigen processing pathway showed that both CB and APB DC subsets possessed all the necessary components of the antigen processing machinery (Ii chain, CLIP and HLA-DM, HLA-DR). However, the levels of expression of these molecules differed between MDCs and PDCs, and between CB and APB. An important finding from this thesis was that CB DCs had a higher surface expression of Ii chain than APB DCs suggesting the lack of degradation of the Ii chain by proteases such as cathepsin S, which could result in the cell surface expression of Ii chain represented as empty class II molecules, devoid of stably bound peptide. This suggestion may be supported by a study which has shown the expression of empty HLA class II molecules, as determined by binding of Ii chain, on human fetal B cells (Garban et al., 1996). Empty HLA class II molecules are developmentally regulated and expressed on immature DCs, and they may possess the ability to bind and present peptide without intracellular processing, which would extend the spectrum of antigens able to be presented by DCs. This would be important in the newborn setting, since it would expand the possibilities for antigen uptake and presentation.

Since most HLA class II-Ii chain complexes transit to the cell surface of DCs *en* route to the endocytic compartment (Saudrais et al., 1998), the complexes may alternatively be functioning as endocytic receptors, capturing antigens for endocytosis and processing by other MHC molecules (Busch et al., 1996). Therefore, the Ii chain expressed by CB DCs may be used and then recycled to the cell surface for further uptake. MHC at the cell surface allows binding and presentation of low-affinity, weakly binding peptides that would otherwise not compete for binding in endosomes, a phenomenon more important in T cell tolerance, which may be a function of CB DCs. Another explanation for the increased surface expression of Ii chain on MDCs may result from the finding that Ii chain targets CD1d molecules to the endocytic pathway and these molecules are preferentially expressed by MDCs (Jayawardena-Wolf et al., 2001). By contrast, intracellular Ii chain expression was reduced in CB supporting the idea that there may be more empty class II molecules at the cell surface of CB cell and this could be tested. The results also suggest a possible delay or defect in the antigen processing capacity of CB DCs. The inefficient processing and subsequent presentation of peptides on the cell surface to T cells may contribute to the dampened immune response as signal 1 (DC-T cell contact through the HLA-peptide complex and TCR) may be delayed or disrupted.

In general, CB DC subsets were phenotypically similar to APB except for the markers of immature/precursor cells, suggesting CB DCs are not fully developed cells and represent DC precursors. Alternatively, CB DC subsets may constitute a separate lineage compared to the adult, and may become redundant following the development of the adult DC subsets.

1.3 Cytokine profile of MDCs and PDCs

The conditioning regimen before transplantation can damage host tissue and induce DC activation, both of which result in cytokine secretion, altering the DC status. Cytokines released by DCs are crucial for interaction with other immune cells (i.e. Th1 and Th2 cells) such as those that initiate cytokine production, and the result of this will be discussed later (see section 6.4). Depending on the DC subset and the

nature of the stimulus, the type of cytokine produced will influence the strength and Th bias of the immune response and therefore, the development of GvHD (Edwards et al., 2002; Steinman and Nussenzweig, 2002). The results of this thesis showed that CB MDCs stimulated with LPS produced proinflammatory cytokines, IL-1β, TNFα and IL-6 at higher concentrations compared to the other cytokines studied, a result which has been shown to contribute to the inflammatory response and GvHD (Tanaka et al., 1995). However, in CBT there is reduced incidence and severity of GvHD, and the fact that the results from this thesis showed that CB MDCs produced cytokines at similar levels to APB MDCs, possibly suggest that the AB serum used in the culture medium may have increased the frequency of cytokine producing CB cells. This scenario has been observed with adult lymphocytes, which were incubated in CB sera to mimic the microenvironment of the fetus. The frequency of cytokine producing adult cells was reduced to that of CB (Lewis et al., 1991; von Freeden et al., 1991; Pirenne-Ansart et al., 1995; Sautois et al., 1997; Chalmers et al., 1998) indicating that the microenvironment during pregnancy may influence the ability of cytokine production, keeping neonatal DCs in a non-responsive state. Differences in the cytokine concentrations can be observed in the sera of CB or APB, which could be responsible for the phenotypic and functional differences between CB and APB cells (Cohen et al., 2000). Therefore, undertaking the same culture experiments, but replacing AB serum with CB serum, may help to explain the observed results.

The above phenomenon may also apply to PDCs, which predominate in CB and were producers of low levels of a range of cytokines following stimulation, whereas, APB PDCs only secreted high concentrations of IFNα. Interestingly, CB PDCs produced significantly lower concentrations of IFNα compared to APB PDCs and CB MDCs. The latter result was particularly surprising, since IFNα is a cytokine known to define PDCs and not MDCs (Cella et al., 1999; Cella et al., 2000). The lack of significant IFNα production by CB PDCs questions the plasmacytoid nature of this DC subset. Therefore, CB PDCs may be a distinct cell subpopulation with similar characteristics to APB PDCs, but specific for neonatal development. Experiments to study this further may include DC differentiation from stem cells, as mentioned previously (section 6.2).

The production of immunoregulatory cytokines by CB DCs *in vivo,* before or after transplantation, may be instrumental in reducing the Th1 response, possibly helping to induce the development of T_{regs}. The cytokines produced may be more efficient in downregulating a cytokine storm observed during GvHD, produced by a CB graft rather than a BM graft. Other factors *in vivo*, such as the cytokines present in the environment can further affect the cytokine repertoire of DCs. For example, G-CSF administered to a donor modulates the cytokine profile of DCs by reducing their secretion of IL-12 (Fagnoni et al., 2004). The reduction in IL-12 secretion would suggest Th2 differentiation or tolerance induction, depending on the cytokine present in the DC environment.

1.4 Functionally distinct DC subsets

The functional characteristics of the CB DC subsets was studied by assessing their endocytic and allostimulatory capacity. The results showed that CB DC subsets were able to endocytose as efficiently as their adult counterparts, indicating no noticeable functional deficit in terms of antigen uptake ability. This illustrates the potential ability of CB DCs to take up soluble material as efficiently as APB DC subsets. As PDCs predominate in CB, endocytosis may be limited in this cell source and antigens may be taken up less efficiently. Future studies may be conducted to assess the ability of CB DC subsets to endocytose and process nominal antigen.

The allostimulatory capacity of CB MDCs and PDCs was then investigated in an MLR assay which has been shown to be a useful *in vitro* predictor of GvHD (Jordan and Ritter, 2002). The reduced allostimulatory capacity of CB DCs and in particular MDCs, compared to APB MDCs correlates well with the low level of HLA class II expression reported in this thesis. As HLA class II expression is important in peptide presentation, reduced levels of these molecules in CB would suggest that less peptide, i.e. minor histoincompatibility antigen (mHAg), could be presented via the indirect pathway. It has also been reported that neonatal DCs are deficient in the upregulation of HLA class II and costimulatory molecules, and therefore, have a limited capacity to promote T cell proliferation (Muthukkumar et al., 2000). Hence,

molecules involved in antigen processing, and cell surface markers involved in DC-T cell contact contribute to the modulation of the immune response. Studying the expression of HLA and costimulatory molecules following activation would be help to confirm other published results (Muthukkumar et al., 2000).

The capacity of PDCs to induce Th1 (Cella et al., 2000), Th2 (Grouard et al., 1997; Rissoan et al., 1999) and T_{reg} cells (Wakkach et al., 2003; Bilsborough et al., 2003) may depend on the signals that induce their activation and maturation (Liu et al., 2001c). Studies have shown that PDCs play an important role in tolerance induction (Gilliet and Liu, 2002) and also the generation and function of $CD4^+CD25^+$ T_{regs} from $CD4^+CD25^-$ T cells, therefore, suppressing T cell proliferation (Moseman et al., 2004). This could also help to explain the reduced allostimulatory capacity of CB DCs observed in this thesis. CB DCs may operate via the promotion of donor derived $CD4^+CD25^+$ T_{regs}, which have been shown to suppress GvHD in other murine models and in tolerance induction to allo HSCT (Taylor et al., 2001; Taylor et al., 2002; Cohen et al., 2002; Hoffmann et al., 2002; Trenado et al., 2003). Injection of allogeneic immature DCs also induced alloantigen-specific hyporesponsiveness of T cells *in vivo* (Dhodapkar et al., 2001). Therefore, targeting steady state DCs with antigen or introducing a Th2 environment *in vivo* can lead to T_{reg} induction.

An interesting finding was highlighted by the results using fresh and frozen cells. Frozen-thawed CB DCs had a reduced allostimulatory capacity compared to fresh CB DCs. This reduction may be the result of the freeze-thaw process, possibly inducing DC inactivation or due to DC apoptosis following DC isolation or during culture. In either case, it may suggest CB DCs are more susceptible than APB DCs to environmental changes. Therefore, measuring apoptotic signals following thawing, isolation and during the culture period may help to establish the viability of DCs and determine whether the results obtained were due to a technical failure or actually the death of CB DCs or functional impairment of DCs. These results are particularly relevant since CB units are routinely frozen prior to transplant, and BM is used fresh, indicating that the freezing process reduces the CB potential for immunoreactivity and GvHD (Cohen et al., 1999). Other results suggest that the

difference between CB and APB cells in the transplant setting, may be due to functional differences and not differences in cell processing (Wang et al., 1998). A previous study has revealed that no deleterious functional effects resulted from freezing CB MNCs with various cell lineages, and primitive progenitors were preserved in the frozen samples (Harris et al., 1994). Harris *et al* stated that the success of a transplant using CB as the source of HSCs relies on the fact that there is ample transfer of HSCs to the patient. Therefore, the functional dynamics of the CB DCs may not be significant. Similar results have been obtained for fresh and frozen CB DCs derived from $CD34^+$ HSCs suggesting that HSCs have a major impact on engraftment even if DCs are not fully functional (Sato et al., 1998; Lewalle et al., 2000; Hori et al., 2004). This may be advantageous in the HSCT setting, where DCs are the major players in GvHD. Therefore, following the freeze-thaw process CB may contain functionally impaired DCs, which are unable to mount an effective Th1 immune response.

Although the use of frozen CB DCs as opposed to fresh CB DCs significantly reduced their allostimulatory capacity, there was no such difference between fresh and frozen APB DCs. The results may be explained by the primitive nature of CB DCs compared to APB DCs. APB DCs may be more robust compared to CB DCs, withstanding the freeze-thaw process, cell separation and culture. In the clinical HSCT setting, since all units of CB are frozen, the diminished allostimulatory capacity of frozen CB DCs compared to APB DCs may also contribute to the reduced severity of GvHD. This is another important factor that could explain the difference in outcome between CBT and BMT.

The cytokine profile following DC-T cell interactions was also studied. As previously stated, factors secreted into the environment include cytokines, which direct Th polarisation following DC-T cell interactions. An infection or malignant disorder leads to the generation of Th1 responses, through IL-2 and IFNγ secretion, facilitating T cell mediated cytotoxicity. In contrast, DCs directing Th2 responses result in the secretion of IL-4, IL-5, IL-6 and IL-10, to help B cells secrete protective antibodies (Banchereau and Steinman, 1998; Tarte and Klein, 1999). During the development of GvHD, T cells are activated and Th1 cytokines released (TNFα, IL-

1β and IL-12) resulting in the destruction of tissues and inducing inflammation through activation of other effector cells. The relative amounts of Th1 and Th2 inducing cytokines are important in determining the extent of GvHD (Ferrara et al., 1996; Blazar et al., 1998). A reduced ability to produce Th1 cytokines would reduce the amplification and effector phases of GvHD not leading to effector cell activation or subsequent tissue damage, instead diminishing the cytokine storm. In this thesis, cytokine secretion following allogeneic stimulation with CB DC subsets resulted in elevated secretion of Th2 cytokines (IL-6, IL-10 and IL-13), which might induce other donor cells to be tolerant (Krenger et al., 1994; Rainsford and Reen, 2002). In addition, similar studies have shown that immature DCs elicit IL-10 producing T_{regs}, which reduce the function of pre-formed Th1 type cells and downregulate the production of proinflammatory cytokines (Jonuleit et al., 2000; Jonuleit et al., 2001; Maloy and Powrie, 2001; Kingsley et al., 2002; Martin et al., 2003; Sundstedt et al., 2003). Therefore, the cytokine milieu DCs are exposed to may influence the incidence of GvHD following HSCT.

1.5 The graft versus host effect

DCs process and present both endogenous and exogenous peptides. In the MHC-matched allogeneic HSCT setting, both donor and recipient DCs present endogenous donor minor histocompatibility antigens (mHAgs) and major antigens such as HLA-DP and/or virally derived peptides (Kato et al., 1991; Petersdorf et al., 1993; Loiseau et al., 2002), modulating T cell activation and/or tolerance induction. The HLA class II antigen presentation pathway incorporates primarily exogenous antigen, facilitating the presentation of host-derived mHAgs by donor-derived APCs. The presentation of exogenous peptides also occurs on HLA class I (cross presentation) (Carbone and Bevan, 1990). However, it is insufficient in initiating GvHD when CD8 cells are placed in an mHAg-mismatched model (Shlomchik et al., 1999). Therefore, professional APCs, such as DCs, are important in initiating a GvH effect and were studied in this thesis.

In terms of allorecognition, recipient DCs were initially shown to play a role in GvHD (Shlomchik et al., 1999; Sato et al., 2003). The presence of donor blood DCs

of PDC origin and loss of recipient DCs were found in patients with *c*GvHD (Clark et al., 2003). The results from this thesis showed the predominance of PDCs in CB possibly suggesting reduced *a*GvHD and increased *c*GvHD. Therefore, the subset distribution and DC origin after transplant may also play a role in *c*GvHD. Since *c*GvHD occurs later than *a*GvHD, and engraftment has already taken place, donor-derived DCs provide a more important contribution. Both *a*GvHD and *c*GvHD differ with regards to the relative roles of CD4 and CD8 T cells, which differ in their APC requirements. CD4 cells have been shown to be important in *c*GvHD, whereas, CD8 cells mediate *a*GvHD, in murine models (Hamilton, 1987; Shlomchik et al., 1999). The suggestion of donor DC involvement in *a*GvHD was introduced more recently (Matte et al., 2004). Although host DCs are involved in driving *a*GvHD, specific donor DCs may suppress or limit *a*GvHD. A study showed high numbers of donor PDCs, or recipient blood PDCs within the first few months of transplant, to be associated with a lower incidence of *a*GvHD (Reddy et al., 2004; Mohty et al., 2005). An explanation for this may be the ability of PDCs to induce Th2 immunity, which has been shown to reduce GvHD incidence and severity (Pan et al., 1995). Since, CB is rich in PDCs as shown from the results of this thesis and by others (Sorg et al., 1999; Borras et al., 2001), the reduced GvH effect following CBT may be a result of donor CB PDCs inducing Th2 responses or tolerance favouring donor engraftment. Instead of modulating the immune properties of APB DCs, CB DCs may be used as an alternative source of HSCs.

1.6 Conclusion

The results from this thesis have shown PDCs are the major DC subpopulation in CB, with CB DCs preferentially expressing markers of immature/precursor cells and could represent a separate lineage. There is a reduced allostimulatory capacity of fresh and frozen CB DCs, and preferential secretion of Th2 cytokines following DC stimulation and allogeneic stimulation. All these results suggest that CB DCs display distinct characteristics compared to APB, which will encourage the reduced development of GvHD, and possibly mimic the tolerogenic environment encountered during pregnancy. However, naïve CB DCs can be stimulated to express equivalent functions to that of APB DCs. The phenotype of CB cells in

some *in vitro* experiments may be bypassed *in vivo* due to a more physiological environment. Therefore, CB can be used as an effective alternative HSC source. CB MDCs and PDCs may be potential candidates to regulate the development of GvHD. In summary, from this thesis we have obtained a better understanding of the DC subsets present in CB, and the results may partially explain the reduced incidence and severity of GvHD following CBT compared to BMT.

1.7 Future work

The study of the phenotypic and functional characteristics of CB DC subsets has revealed several important findings. However, there are several future directions resulting from the work undertaken, that could be investigated.

- It would be advantageous to immunophenotype and compare the expression of markers specific for CB and APB DC subsets when freshly isolated and following the freeze-thaw process, as this may have affected the phenotype and so influenced the outcome of the allogeneic response.

- Another interesting finding was the expression of HLA-G by CB DCs and not by their adult counterparts. The secretion of membrane and soluble HLA-G during an MLR could be studied. Blocking experiments may also be used to determine the involvement of HLA-G in the immunosuppressive effects observed following allostimulation.

- Studying the functional characteristics of the CB DC subsets during an MLR may determine if DCs undergo apoptosis during culture with T cells or induce T_{reg} development.

- The endocytic capacity could be further studied by assessment of other receptors, such as DEC-205 on MDCs and BDCA2 expressed by PDCs. The phagocytic capacity using latex beads would be another perspective to study antigen uptake. Studies showing the efficient phagocytic capacity of MDCs but

not of PDCs isolated from APB have not been extended to CB DCs. The uptake of nominal antigen may also be another area of interest.

- *In vitro*, CB DCs are highly purified cells, based on specific marker expression with a phenotypic and functional similarity to adult cells. *In vivo*, there are other cell types present and their location (tissue) and environment (CB sera) differ. There may be factors in AB sera that are absent in CB sera that enhance proliferative responses. Therefore, studying CB DCs in their own environment of CB sera and treating APB DCs in the same manner, may help determine whether there are factors present in CB sera which may alter DC characteristics.

- The study of the presence of CB DC subsets and their functional characteristics pre- and post-transplant from patients receiving either CB or BM, would reveal important information regarding engraftment and cell recovery.

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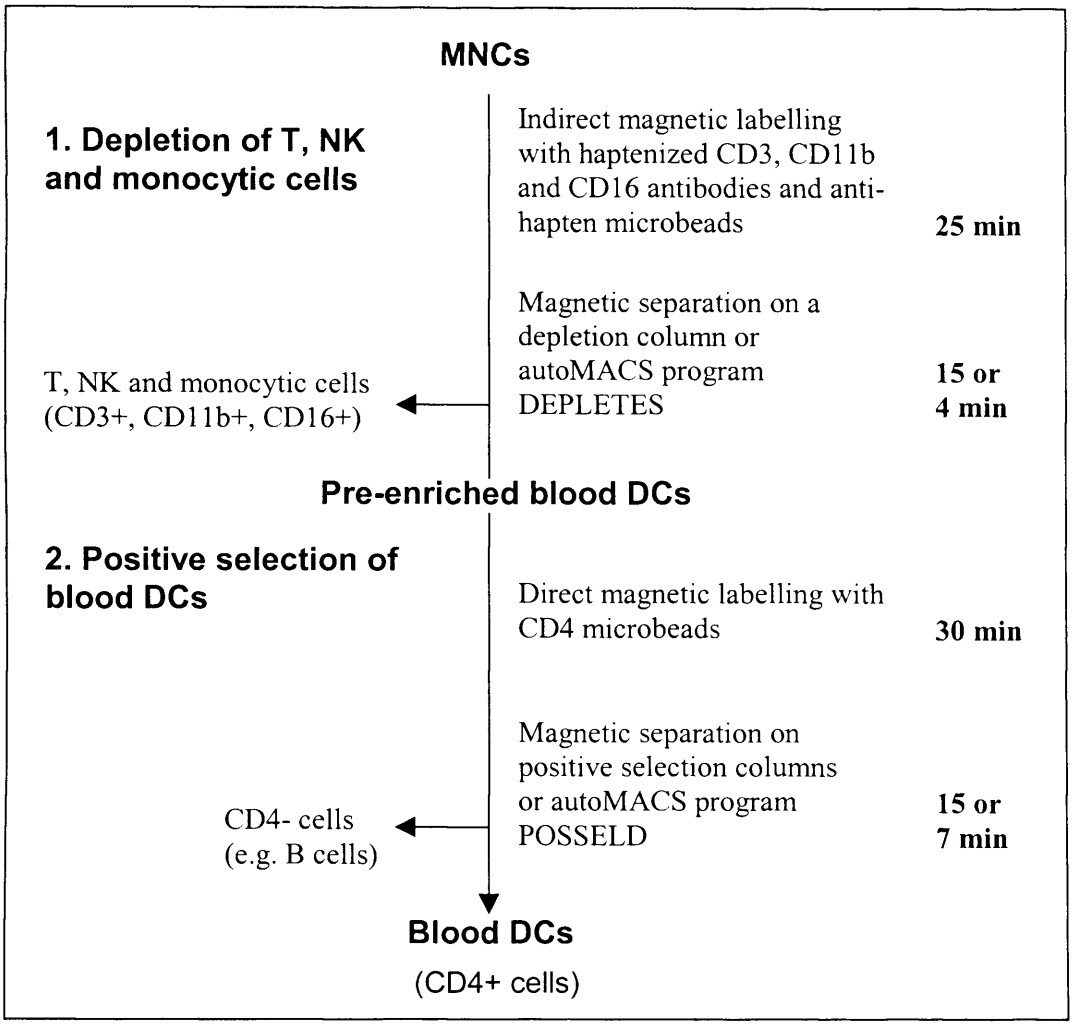
Appendices

Appendix 1. Materials required for methods

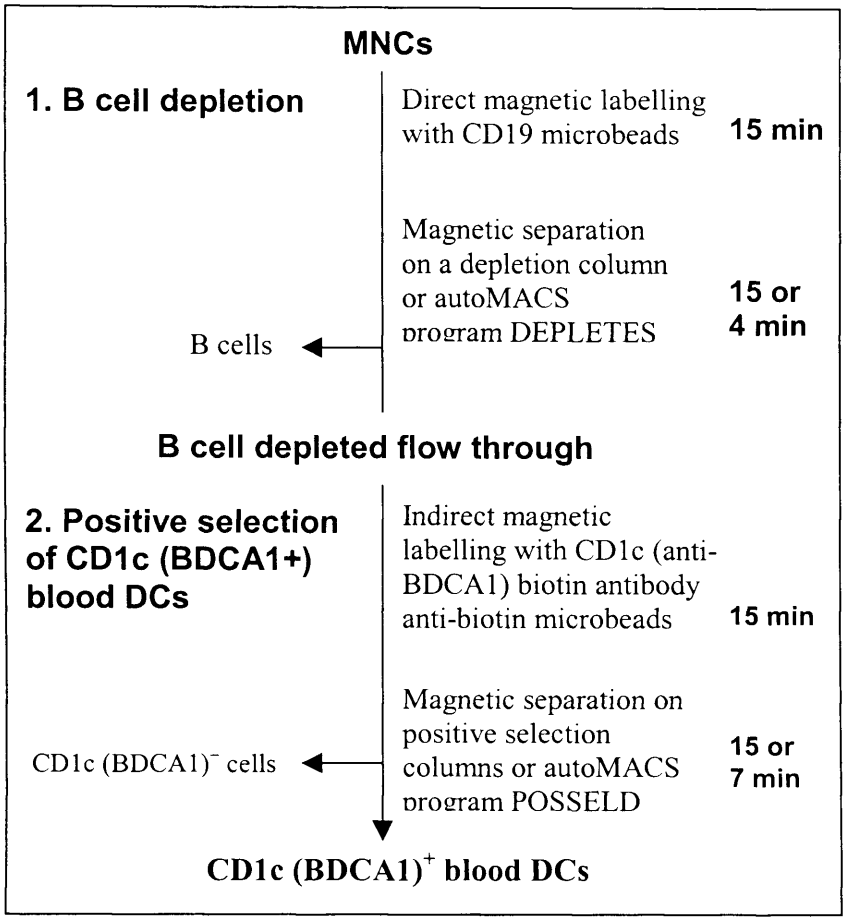
A panel of monoclonal antibodies used for immunophenotyping of dendritic cell subsets.

Monoclonal antibody	Specificity		Company
HLA-CLASS I	FITC	All nucleated cells	BD Pharmingen
HLA-E	FITC		Exbio
HLA-G	FITC	Cytotrophoblasts	Exbio
HLA-DM	PE	Class II	BD Pharmingen
HLA-DP	FITC	APCs	Cymbus
HLA-DQ	FITC	APCs	BD Pharmingen
HLA-DR	PerCP	APCs	BD Biosciences
CD1a	FITC	DCs	BD Pharmingen
CD1c (BDCA1)	APC	MDCs	Miltenyi Biotec
CD1d	PE	Lipid antigens	BD Pharmingen
CD4	PE	DCs	BD Biosciences
CD8	FITC	Murine DCs	BD Pharmingen
CD11a (LFA-1)	PE	Adhesion molecule	BD Pharmingen
CD11b (Mac-1)	PE	MDC	BD Pharmingen
CD11c	APC	MDC	BD Pharmingen
CD13	FITC	MDC	Beckman Coulter
CD14	FITC/PE	Monocytes, MDCs	BD Pharmingen
CD16	FITC/PE	MDC	BD Pharmingen
CD19	FITC/PE	B cells	BD Pharmingen
CD33	PE	MDCs	BD Biosciences
CD34	FITC	Haematopoeitic cells	BD Biosciences
CD40	PE	Mature DC	BD Pharmingen
CD45	FITC	Common leucocyte Ag	BD Biosciences
CD45RA	FITC	Naïve T cell, PDCS	BD Biosciences
CD45RO	PE	Memory T cell, MDC	BD Biosciences
CD74 (Ii chain)	FITC	APCs	BD Pharmingen
CD80	PE	Mature DCs	BD Pharmingen
CD83	FITC	Mature DCs	BD Pharmingen
CD86	PE	DC	BD Pharmingen
CD123	PE	PDCs	BD Pharmingen
CD133 (AC141)	PE	Primitive progenitors	Miltenyi Biotec
CD206 (MMR)	FITC	DC	BD Pharmingen
CD209 (DC-SIGN)	PE	DC	BD Pharmingen
CD303 (BDCA2)	APC	PDCS	Miltenyi Biotec
CD304 (BDCA4)	APC	PDCs	Miltenyi Biotec
CLIP (CerCLIP)	FITC	APCs	BD Pharmingen
Isotype controls	All	Negative controls	BD Biosciences
Lin1 (CD3,CD14, CD19,CD20, CD56)	FITC	Haematopoietic lineage markers	BD Biosciences

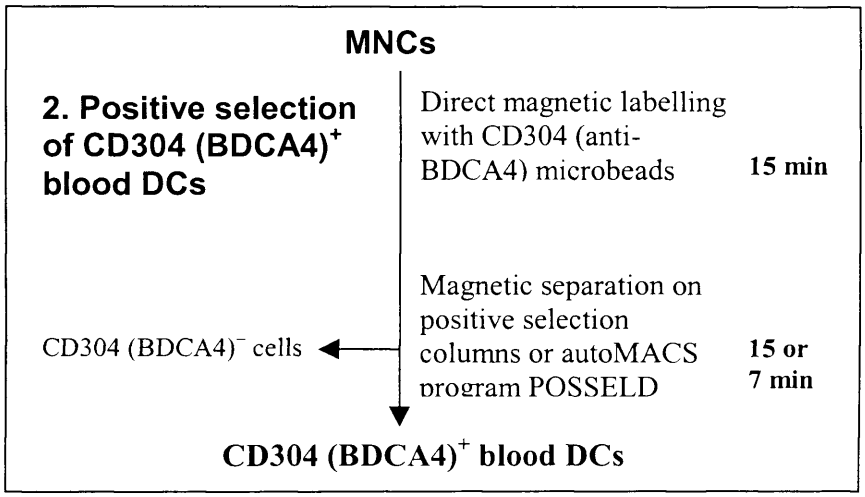
A flow chart demonstrating the method for the isolation of 'total AB DCs'.



(a) A flow chart demonstrating the method for the isolation of MDCs.



(b) A flow chart demonstrating the method for the isolation of PDCs.



The list of primers used for determining the presence of HLA-G isoforms.

Primer	Sequence (5' - 3')
G2-5'	TCCATGAGGTATTTCAGCGC
GC-2a	GGCTGGTCTCTGCACAAAGAGA
G257F	GGAAGAGGAGACACGGAACA
HLA-GEx5	ACAACCAGGCCAGCAACG
G526F	CCAATGTGGCTGAACAAAGG
i4b	AAAGGAGGTGAAGGTGAGGG
β actin – F	ATCTGGCACCACACCTTCTACAATGAGCTGCG
β actin – R	CGTCATACTCCTGCTTGCTGATCCACATCTGC

The primers were paired as G2-5' and GC-2a for the first round of PCR. For nested PCR, the forward and reverse primer pairings were G257F and GEx5, G526F and GEx5, G257F and Gi4b.

The PCR condition for the various primer sets used to detect HLA-G isoforms.

Primer set	PCR conditions
1st round PCR: G2-5' and GC-2a	Initial: 5 min, 94°C
2nd round nested PCR: G257F and HLA-GEx5	40 cycles: 1 min, 94°C
G526F and HLA-GEx5	1 min 30 sec, 61°C
	2 min, 72°C
G257F and i4b	Final: 10 min, 72°C

Appendix 2. Immunophenotyping results of CB and APB MDCs and PDCs.

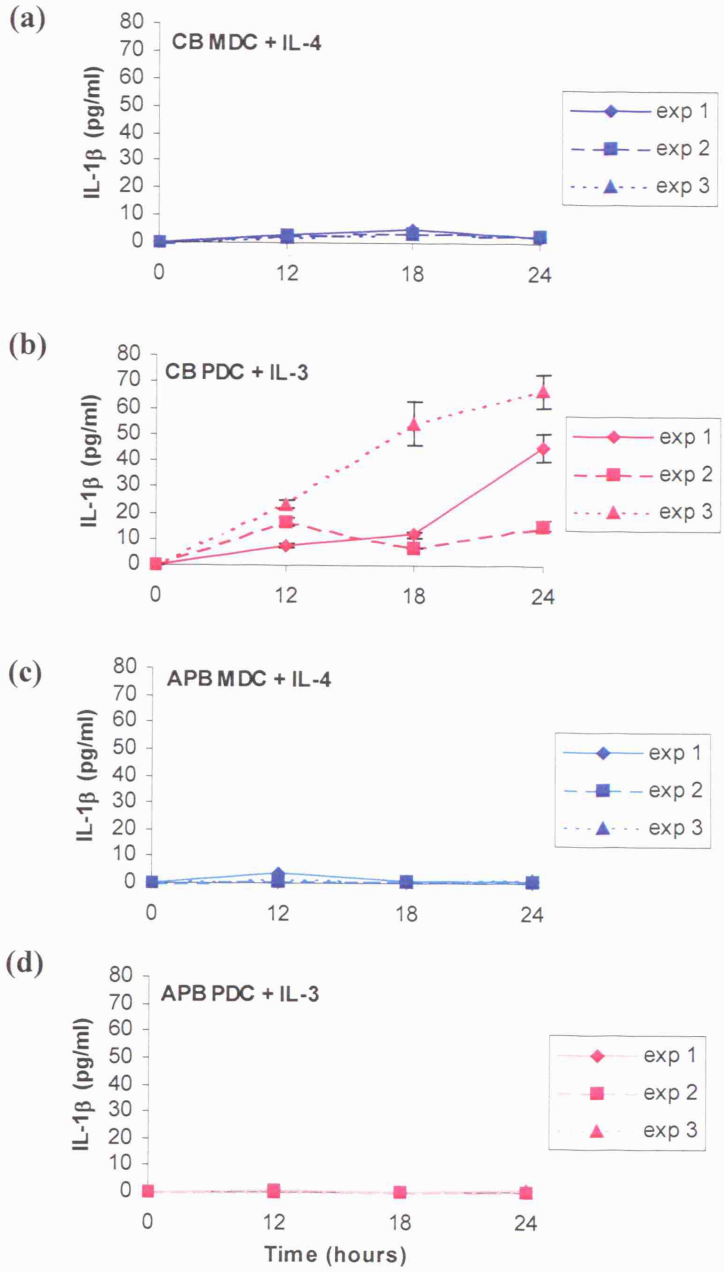
The mean and standard deviation of the percentage of DC subsets expressing various molecules.

Molecule	CB		AB	
	MDC	PDC	MDC	PDC
HLA-A,B,C	98.3 ± 0.8	99.9 ± 0.3	98.9 ± 1.5	100.0 ± 0.0
HLA-E	0.6 ± 0.7	0.3 ± 0.4	0.5 ± 0.6	0.0 ± 0.0
HLA-E (IC)	53.4 ± 18.2	79.5 ± 0.1	16.6 ± 2.9	39.8 ± 6.8
HLA-G	1.6 ± 1.2	1.0 ± 1.3	0.5 ± 0.5	0.1 ± 0.2
HLA-G (IC)	61.3 ± 31.6	48.7 ± 42.1	17.6 ± 13.3	10.9 ± 5.5
HLA DR	95.7 ± 4.4	96.2 ± 3.0	99.1 ± 0.9	96.9 ± 3.9
HLA-DR (IC)	99.8 ± 0.2	98.9 ± 0.4	98.8 ± 1.6	99.5 ± 0.4
HLA-DQ	94.1 ± 2.8	11.4 ± 4.3	96.5 ± 1.9	27.8 ± 7.0
HLA-DP	60.3 ± 17.1	4.0 ± 5.7	94.1 ± 4.0	18.4 ± 26.7
HLA-DO	0.5 ± 0.3	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
HLA-DO (IC)	28.0 ±	0.6 ± 0.0	10.8 ± 4.1	1.2 ± 1.4
CD1a	1.8 ± 1.3	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
CD1c (BDCA1)	93.0 ± 0.6	0.0 ± 0.0	97.1 ± 2.2	0 ± 0.0
BDCA2	0.0 ± 0.0	87.0 ± 0.0	0.0 ± 0.0	97.7 ± 2.2
CD1d	87.5 ± 11.7	0.0 ± 0.0	98.3 ± 1.5	0.2 ± 0.3
CD4	90.8 ± 1.9	99.9 ± 0.1	98.9 ± 1.5	98.6 ± 2.8
CD8	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CD11a (LFA-1)	98.9 ± 1.1	100 ± 0.0	98.6 ± 1.7	98.6 ± 1.0
CD11b	36.2 ± 18.2	0.0 ± 0.0	48.6 ± 12.5	0.3 ± 0.5
CD11c	90.6 ± 15.1	0.0 ± 0.0	95.0 ± 1.8	0.1 ± 0.2
CD13	10.1 ± 13.7	2.6 ± 4.2	24.1 ± 9.5	0.3 ± 0.7
CD14	34.9 ± 20.3	0.5 ± 0.4	28.7 ± 7.3	0.3 ± 0.6
CD16	3.3 ± 0.8	0.0 ± 0.0	10.6 ± 3.1	0.9 ± 1.6
CD19	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
CD33	92.1 ± 3.2	17.1 ± 14.2	96.4 ± 4.0	47.2 ± 19.0
CD34	1.3 ± 0.9	16.8 ± 14.5	0.0 ± 0.0	0.0 ± 0.0
CD40	4.4 ± 4.9	12.2 ± 10.3	6.5 ± 4.0	0.5 ± 0.8
CD45	97.8 ± 3.1	99.1 ± 1.7	95.8 ± 5.4	99.0 ± 1.1
CD45RA	85.2 ± 13.5	100 ± 0.0	34.3 ± 25.3	98.4 ± 1.8
CD45RO	22.3 ± 6.4	0.1 ± 0.1	69.8 ± 5.4	0.5 ± 0.8
CD80	1.0 ± 0.1	0.3 ± 0.5	0.1 ± 0.3	0.0 ± 0.1
CD83	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.2	0.0 ± 0.1
CD86	90.3 ± 1.5	6.2 ± 3.2	89.5 ± 5.7	10.0 ± 4.8
CD123	90.7 ± 3.4	96.1 ± 3.1	80.9± 7.2	99.2 ± 0.6
CD133	9.8 ± 16.3	2.3 ± 2.3	0.5 ± 0.7	1.0 ± 1.2
CD206 (MR)	0.4 ± 0.8	0.3 ± 0.7	5.7 ± 4.1	0.2 ± 0.3
CD209 (DC-SIGN)	2.4 ± 2.3	0.8 ± 1.5	4.4 ± 1.0	0.4 ± 0.4
FAS (CD95)	43.0 ± 8.7	0.2 ± 0.2	43.6 ± 31.3	0.0 ± 0.0
CLIP	3.7 ± 3.0	0.3 ± 0.4	10.5 ± 7.6	0.5 ± 0.4
CLIP (IC)	3.3 ± 3.5	0.4 ± 0.6	12.3 ± 15.2	0.2 ± 0.3
HLA-DM	0.3 ± 0.2	0.1 ± 0.1	0.5 ± 0.7	0.0 ± 0.0
HLA-DM (IC)	74.1 ± 21.3	61.2 ± 25.3	84.7 ± 18.7	74.9 ± 27.7
Ii chain	46.2 ± 18.6	9.5 ± 6.0	12.7 ± 2.0	0.3 ± 0.3
Ii chain (IC)	83.3 ± 14.0	93.8 ± 5.3	91.6 ± 12.4	98.9 ± 1.4

The mean and standard deviation of the level of expression (MFI) of DC subsets expressing various molecules.

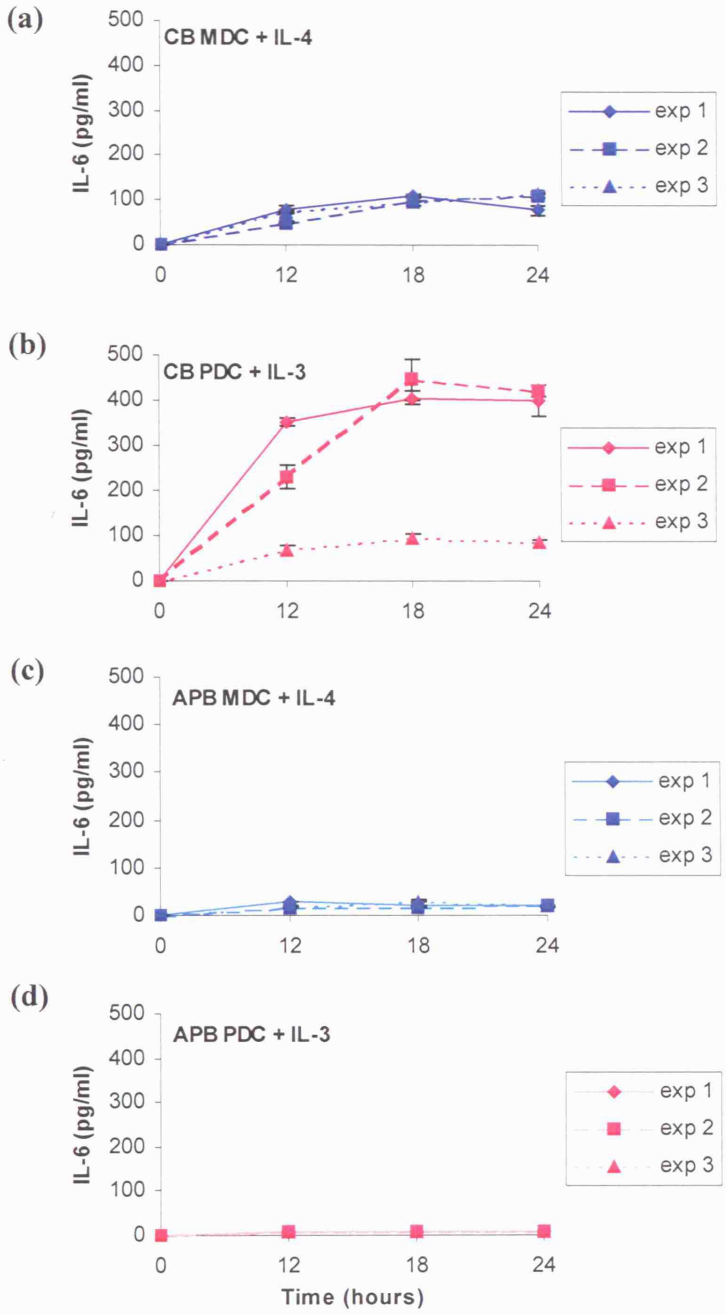
Molecule	CB		AB	
	MDC	PDC	MDC	PDC
HLA-A,B,C	312.9 ± 8.2	103.4 ± 1.9	513.8 ± 184	287 ± 168
HLA-E	N/A	N/A	N/A	N/A
HLA-E (IC)	12.5 ± 1.2	13.9 ± 0.8	20.8 ± 0.7	23.9 ± 2.8
HLA-G	N/A	N/A	N/A	N/A
HLA-G (IC)	22.5 ± 12.1	12.1 ± 7.9	16.7 ± 4.3	5.6 ± 6.6
HLA DR	205.5 ± 41.8	92.7 ± 53.4	432.7 ± 41	182 ± 101
HLA-DR (IC)	238.6 ± 74.1	73.5 ± 27.9	522.4 ± 148	104.5 ± 57
HLA-DQ	60.9 ± 42.8	8.8 ± 9.7	65.7 ± 15.6	9.6 ± 3.8
HLA-DP	17.3 ± 5.2	2.4 ± 3.3	81.4 ± 6.0	19.0 ± 32
HLA-DO	N/A	N/A	N/A	N/A
HLA-DO (IC)	9.4 ± ?	4.0 ±	13.8 ± 1.0	10.4 ± 1.8
CD1a	N/A	N/A	N/A	N/A
CD1c (BDCA1)	74.2 ± 13.2	N/A	89.4 ± 8.5	N/A
BDCA2	N/A	151.1 ±	N/A	226 ± 77.2
CD1d	113.7 ± 77.3	2.3 ± ?	184.5 ± 67.7	6.6 ± 6.1
CD4	77.4 ± 11.1	146.2 ± 24.8	205.1 ± 80.8	510.4±131
CD8	N/A	N/A	N/A	N/A
CD11a (LFA-1)	688.9 ± 219.8	296.7 ±	1056 ± 71.1	613 ± 77.3
CD11b	19.7 ± 0.5	0.0 ± 0.0	17.3 ± 10.3	N/A
CD11c			49.7 ± 15.6	N/A
CD13	5.2 ± 3.5	2.9 ± 4.0	10.0 ± 2.4	N/A
CD14	143.2 ± 73.9	N/A	10.0 ± 1.9	N/A
CD16	N/A	N/A	7.4 ± 3.8	N/A
CD19	N/A	N/A	N/A	N/A
CD33	137.8 ± 74.1	6.9 ± 2.1	350.5 ± 170	21.5 ± 24
CD34	8.1 ± 4.7	6.2 ± 5.0	N/A	N/A
CD40	5.4 ± 6.6	6.3 ± 7.2	6.9 ± 1.9	N/A
CD45	565 ± 358.8	195.4 ± 154	734.3 ± 191	560 ± 107
CD45RA	65.5 ± 46.5	563.6 ± 124	24.0 ± 16.0	493 ± 453
CD45RO	7.2 ± 4.4	N/A	19.8 ± 13.9	N/A
CD80	N/A	N/A	N/A	N/A
CD83	N/A	N/A	N/A	N/A
CD86	77.8 ± 16.5	N/A	56.9 ± 26.6	N/A
CD123	69.9 ± 21.2	1110 ± 321	39.1 ± 11.8	750 ± 319
CD133	N/A	N/A	N/A	N/A
CD206 (MR)	N/A	N/A	4.1 ± 1.3	N/A
CD209 (DC-SIGN)	N/A	N/A	4.2 ± 0.4	N/A
FAS (CD95)	10.3 ± 0.5	N/A	11.4 ± 7.3	N/A
CLIP	6.4 ± 6.0	5.0 ± 2.4	4.8 ± 0.0	N/A
CLIP (IC)	8.3 ± 3.2	4.9 ± 1.3	7.3 ± 5.4	3.2 ± 2.7
HLA-DM	N/A	N/A	N/A	N/A
HLA-DM (IC)	30.9 ± 9.0	16.1 ± 4.8	51.6 ± 32.1	28.9 ± 0.3
Ii chain	16.8 ± 5.1	7.7 ± 3.5	6.5 ± 1.5	N/A
Ii chain (IC)	254.5 ± 238.6	642.8 ± 281	274.1 ± 252	894 ± 450

Appendix 3. The kinetic profile of steady state CB and APB MDCs and PDCs.



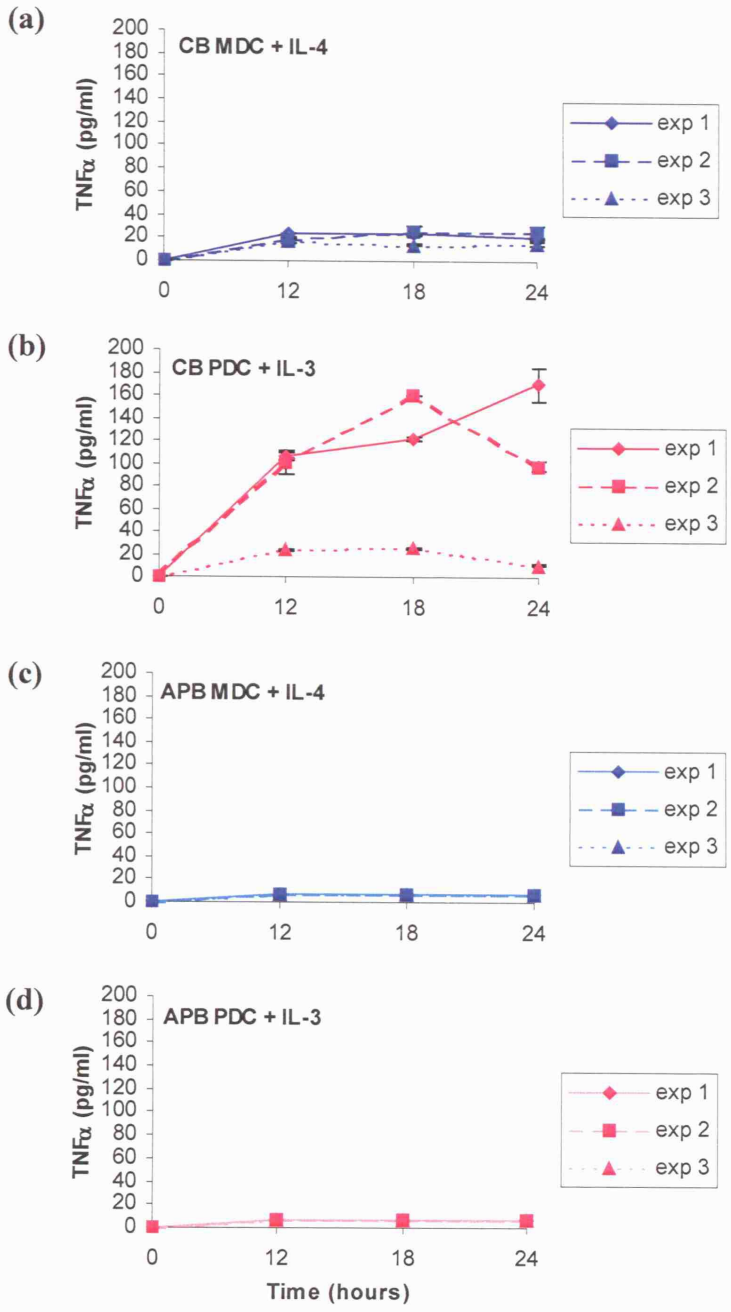
Secretion of IL-1β in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for IL-1β detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4: (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d) APB PDCs + IL-3.



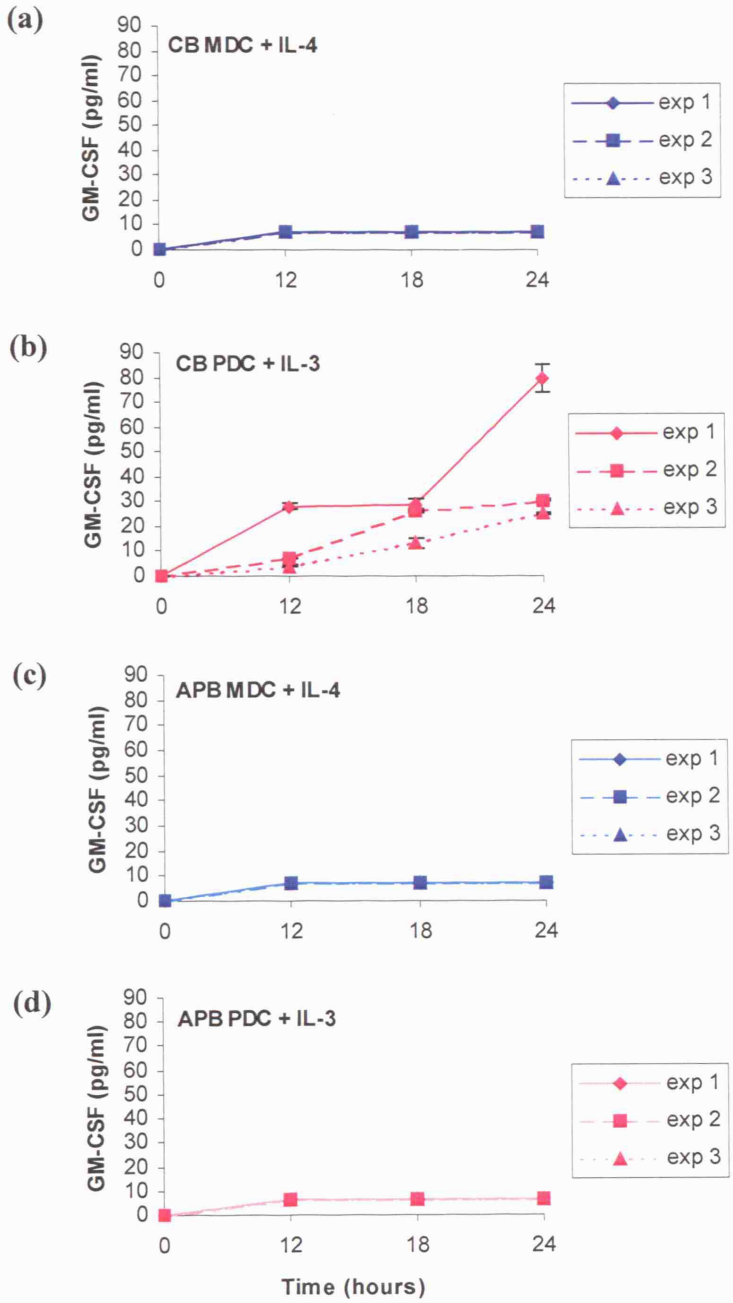
Secretion of IL-6 in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for IL-6 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4: (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d) APB PDCs + IL-3.



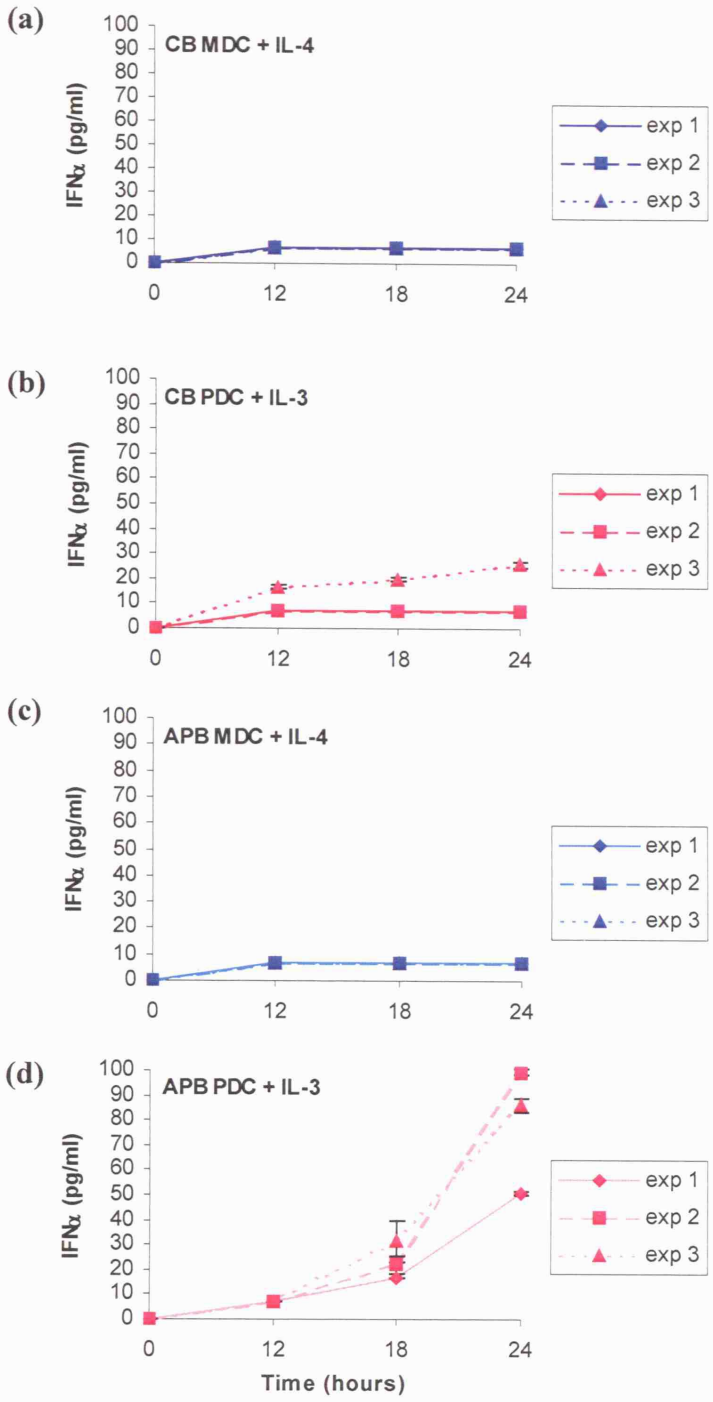
Secretion of TNFα in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively over a 24 hr time period before removing supernatants for TNFα detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4 (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d)APB PDCs + IL-3.



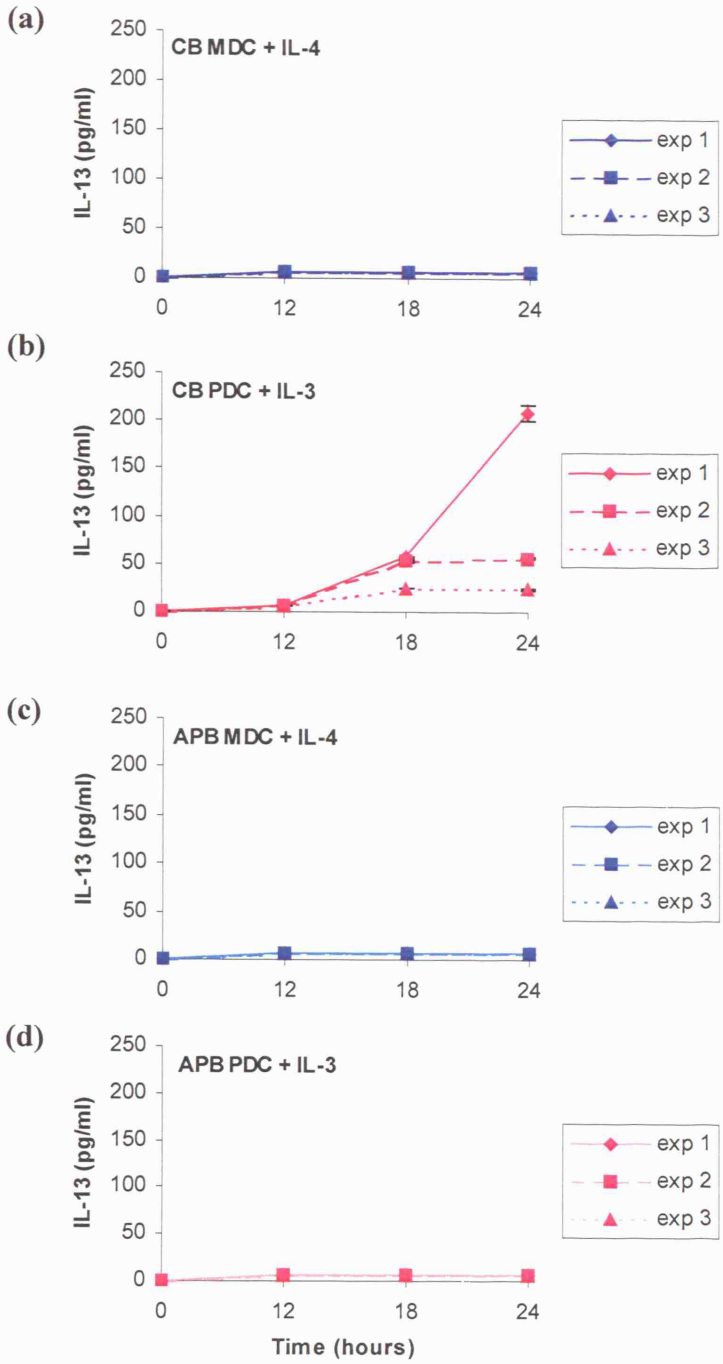
Secretion of GM-CSF in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for GM-CSF detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4 (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d)APB PDCs + IL-3.



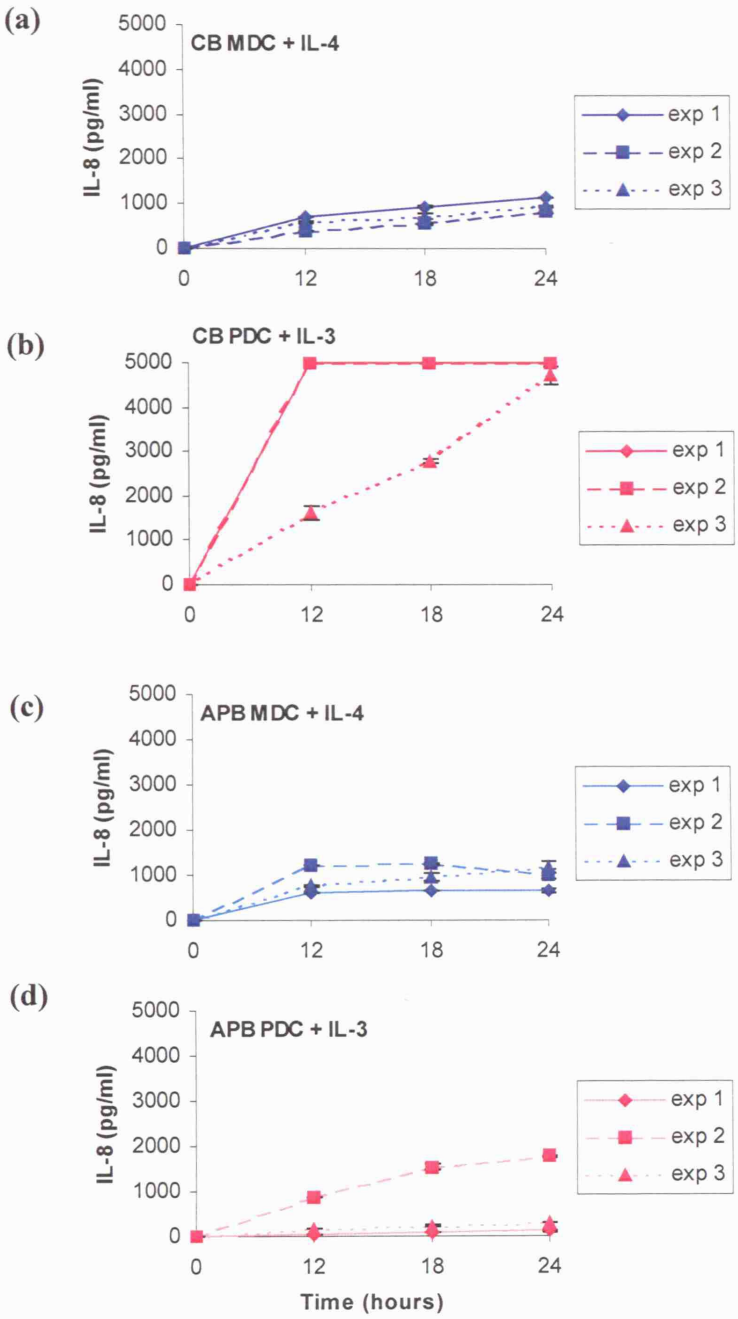
Secretion of IFNα in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for IFNα detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4 (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d)APB PDCs + IL-3.



Secretion of IL-13 in the steady state.

MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for IL-13 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4 (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d) APB PDCs + IL-3.

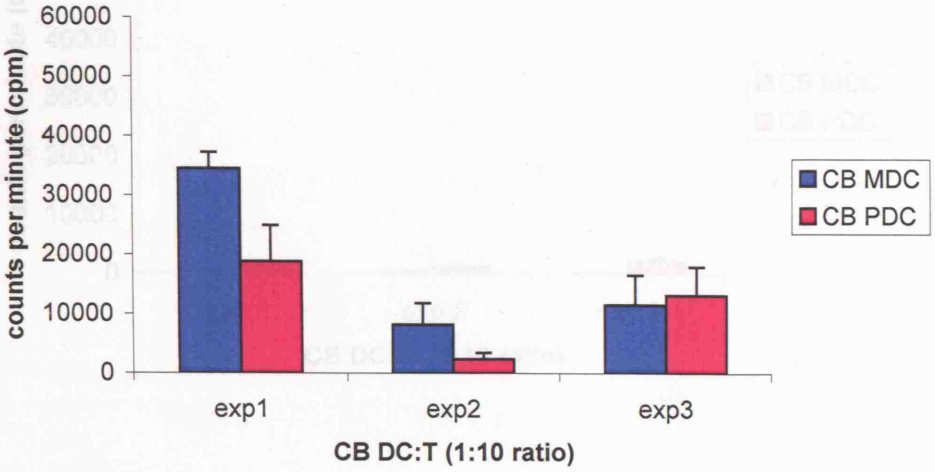


Secretion of IL-8 in the steady state.

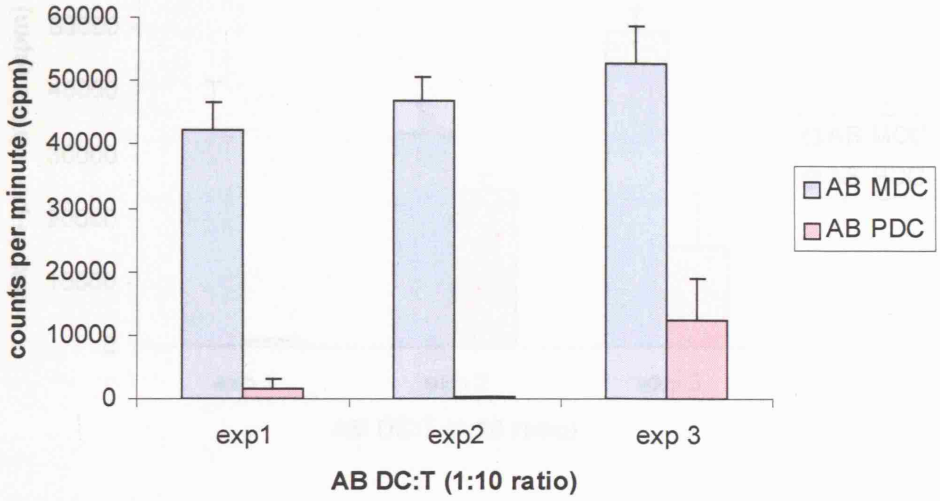
MDCs and PDCs were isolated using the MACS technique and cultured with IL-4 and IL-3, respectively, over a 24 hr time period before removing supernatants for IL-8 detection by bead-based Luminex® technology. The graphs show (a) CB MDCs + IL-4 (b) CB PDCs + IL-3 (c) APB MDCs + IL-4 and (d) APB PDCs + IL-3.

Appendix 4. A summary of the functional studies on MDCs and PDCs.

(a)



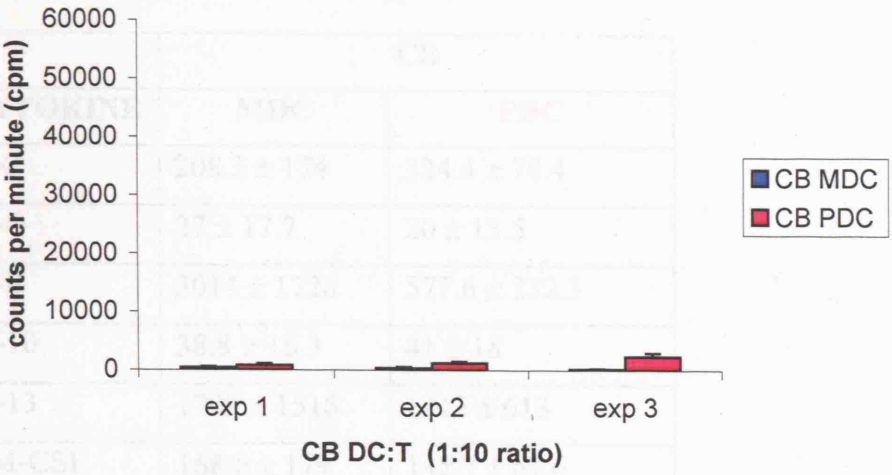
(b)



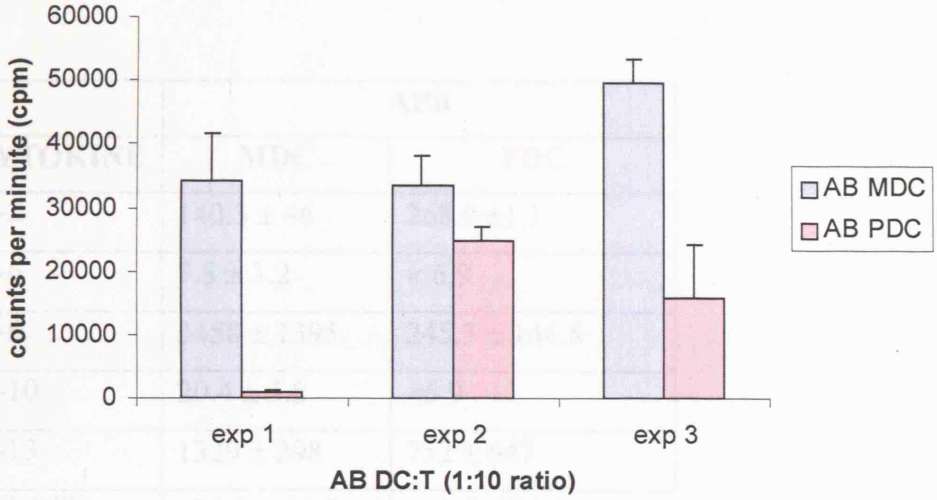
The allostimulatory capacity of freshly isolated myeloid and plasmacytoid dendritic cells at a 1:10 ratio.

The allostimulatory capacity of MDCs and PDCs isolated from fresh (a) CB and (b) AB MNCs, at the optimum ratio of 1:10, stimulator to responder (DC-T). The results show the mean ± SD of three independent experiments (n = 3).

(a)



(b)



The allostimulatory capacity of frozen myeloid and plasmacytoid dendritic cells at a 1:10 ratio.

The allostimulatory capacity of MDCs and PDCs isolated from frozen (a) CB and (b) AB MNCs, at the optimum ratio of 1:10, stimulator to responder (DC-T). The results show the mean ± SD of three independent experiments (n = 3). The percentage viability following the freeze-thaw process was between 90-95%.

The cytokine profile following stimulation of allogeneic T cells with CB and APB DC subsets at a ratio of 1:10. N.B. All results are in pg/ml.

	CB	
CYTOKINE	**MDC**	**PDC**
IL-2	208.3 ± 174	324.4 ± 70.4
IL-6	27 ± 17.7	20 ± 13.5
IL-8	3014 ± 1726	577.6 ± 232.3
IL-10	38.8 ± 16.3	41 ± 18
IL-13	1799 ± 1518	1413 ± 613
GM-CSF	168.6 ± 179	145.7 ± 62.6
IFNγ	587.8 ± 776.7	719 ± 524
TNFα	47.4 ± 52.3	< 6.9

	APB	
CYTOKINE	**MDC**	**PDC**
IL-2	140.3 ± 46	268.9 ±1.1
IL-6	7.8 ± 3.2	< 6.9
IL-8	3458 ± 1395	245.3 ± 144.8
IL-10	20.4 ± 5.8	<6.9
IL-13	1329 ± 298	752 ± 647
GM-CSF	170.2 ± 43.7	109.7 ± 33.9
IFNγ	837 ± 69.9	275 ± 191
TNFα	34.3 ± 28.3	< 6.9

Relevant publications

Gómez J, Borràs FE, Singh R, Rajananthanan P, English N, Knight SC and Navarrete CV. *Differential upregulation of HLA-DM, invariant chain and CD83 on myeloid and plasmacytoid dendritic cells from peripheral blood.* Tissue Antigens, 2004, 63 (2): 149-157.

Mar Naranjo MA, Fernandez MB, Singh R, Navarrete CV, Pujol-Borell,R and Borràs FE. *Primary alloproliferative Th1 response induced by immature plasmacytoid dendritic cells in collaboration with myeloid DCs.* American Journal of Transplantation, 2005, 5: 2838.

Singh R and Navarrete CV. *The characterisation of dendritic cell subsets* (in preparation).

Invited oral presentations

R Singh, J Gomez, F Borras, I Karakikes, N Fernandez and CV Navarrete. Phenotypic and functional characterisation of myeloid and plasmacytoid dendritic cell subsets.
ISBT, July 2004.

Rena Singh, Jorge Martinez-Laso and Cristina Navarrete. Expression of HLA-G in CD133+ stem cells and in myeloid dendritic cells from cord blood.
European Immunogenetics and Histocompatibility Conference, April 2005.

R Singh and CV Navarrete. Phenotypic and functional characterisation of myeloid and plasmacytoid dendritic cell subsets from cord blood.
BBTS, December 2005.

Abstracts

Rena Singh, Jesús Gómez, Francesc E. Borràs, Palasingam Rajananthanan, Nicholas English, Stella C. Knight and Cristina V. Navarrete. Differential expression of HLA-DM in cord blood and adult blood dendritic cells subsets.
International Symposium on Dendritic Cells, September 2002.

Rena Singh, Jesus Gomez, Francesc Borras, Cristina Navarrete. Expression of antigen processing related molecules in myeloid and plasmacytoid dendritic cells. **European Macrophage and Dendritic Cell Society, August 2003.**

R.Singh, J.Gomez, S.Brough, P.Rajananthanan and C.V.Navarrete. (2000). Comparison of flow cytometric and radioactive methods in evaluating autologous T cell proliferation against nominal antigen. Eur J Immunogenet. *30*, 310.

R Singh, J Gomez, F Borras, I Karakikes, N Fernandez and CV Navarrete. (2005). Phenotypic and functional characterisation of myeloid and plasmacytoid dendritic cell subsets. Vox Sang. *87*, 8.

Rena Singh, Jorge Martinez-Laso and Cristina Navarrete. (2005). Expression of HLA-G in CD133+ stem cells and in myeloid dendritic cells from cord blood. Gene Imm. *6,* 58.

R Singh and CV Navarrete. Phenotypic and functional characterisation of myeloid and plasmacytoid dendritic cell subsets from cord blood. Transfusion Medicine, 2005, 15 (S1): 22.